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Involvement of AMP-activated Protein Kinase and Phosphoinositide-3-kinase in BLX-1002 induced insulin secretion.

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Abstract

BLX-1002 is an amino acid conjugated, novel small molecule drug, which was shown to benefit to diabetes through lowering blood glucose and improving lipid profile in the circulation in vivo. BLX-1002 has been identified as an interesting drug-candidate for type II diabetes.

Preliminary results from the research group were shown that BLX-1002 enhanced insulin secretion from pancreatic β-cells only at high glucose, but not at non-stimulatory concentrations of the sugar. The mechanisms through which BLX-1002 mediates the effects are still unknown. The purpose of this project was to find out if BLX-1002 affected the activity of AMPK and PI3K, two signalling enzymes believed to play a role in pancreatic β-cell function and diabetic pathogenesis. The method chosen was Western blotting. The experiment was carried out in ob/ob mouse islet cells, once it was confirmed that the experimental set up worked in MIN6 cells. The effect of BLX-1002 on insulin secretion in the presence of PI3K and AMPK inhibitors was also studied.

The results imply that BLX-1002 activates both AMPK and PI3K. In both cases the activation is temporary and followed by an inhibition. The fact that these two enzymes are known to negatively regulate insulin secretion makes these results hard to explain. The present study revealed that when the activity of the enzymes was inhibited, the BLX-1002 induced insulin secretion was abolished. This indicates that the both enzymes are required for BLX-1002 induced insulin secretion.

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List of abbreviations

AMPK – AMP-activated protein kinase

PI3K – Phosphoinositide-3-kinase

BLX-1002 – a candidate drug for treatment of diabetes mellitus

GLUT4 – a glucose transporter

BSA – bovine serum albumin

Introduction

Diabetes mellitus is a disease that affects more than 100 million people all over the world. There are two types of diabetes: type 1 and type 2. In people with type 1 diabetes, the β-cells are damaged and cannot produce sufficient or any insulin. Type 1 diabetes is an autoimmune disease and often starts affecting people when they are young, usually before age 15. Type 1 diabetes patients are thin and are affected with hyperglycaemia, dehydration, excessive urination, hunger and thirst.

Type 2 diabetes is characterised by chronic hyperglycaemia due to increased insulin resistance in combination with relative deficiency in insulin secretion. The decreased sensitivity to insulin is caused by a shortage of insulin receptors and/or decreased activity of the insulin receptors [1]. When the insulin receptors start to fail in a diabetic patient, there will be an increased demand of insulin. At first, the β-cells will compensate for the insulin resistance by increasing the secretion of insulin. This will keep the levels of glucose at a normal level. But after some time, this period of β-cell compensation is followed by β-cell failure. The number and function of β-cells will decline. This will lead to an impairment of the insulin secretion, which in turn lead to hyperglycaemia. [2].

Type 2 diabetes is usually associated with obesity and adult age [1]. Approximately 60% to 90% of the cases with type 2 diabetes appear to be related to obesity [2]. Obesity induced insulin resistance (type 2 diabetes) leads to elevated levels of plasma insulin (in the beginning of the disease), glucose and fatty acids, which in turn create a proinflammatory oxidative environment [3].

BLX-1002

BLX-1002 is a new anti-diabetic drug that appears to protect against β-cell failure and the proinflammatory environment caused by obesity induced insulin resistance. The drug also seems to stimulate insulin release *in vitro* at high, but not at low levels of glucose (fig.1). It lowers the levels of glucose, free fatty acids and cholesterol in animal models and it also has a unique anti-weight gain property. These are properties that would be beneficial to diabetic patients.

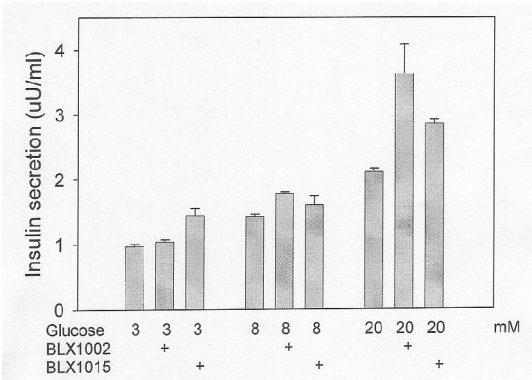


Fig. 1. This is a preliminary result from the Diabetes group at KISÖS, Stockholm. The bar chart represents the insulin secretion from pancreatic β-cells from ob/ob mice (060110-12). The cells were incubated in the presence of BLX-1002 (10μM) or BLX-1015 (an analogue structurally related to BLX-1002, 10μM) or vehicle (DMSO) at three different concentrations of glucose (3mM, 8mM and 20mM) for 20 minutes, at 37℃. BLX-1002 converts to BLX-1015 after oral dosing.

BLX-1002 is an amino acid conjugated, water-soluble molecule with a molecular mass of 500 Dalton. The molecule has no structural resemblance with any existing anti-diabetic compounds. It is non-adipogenic and has no affinity to PPARγ nuclear receptors [4].

Not much is known about the mechanisms through which BLX-1002 mediates its effects. In this project, Western blot was used to study the effects of BLX-1002 on two enzymes involved in the β-cell function: AMP-activated protein kinase (AMPK) and Phosphoinositide-3-kinase (PI3K). An additional study on BLX-1002 induced insulin secretion was also performed to see how PI3K and AMPK inhibitors affect BLX-1002 induced insulin secretion.

BLX-1002 has completed safety toxicity pre-clinical studies in the US in both lower order animals like rodents and higher order animals like dogs. It has also completed safety studies in healthy and diabetic patients.

AMPK

AMPK is an energy sensor that regulates cellular metabolism. The enzyme controls whole body homeostasis through regulation of metabolism in peripheral tissues, such as liver, skeletal muscle, adipose tissue and pancreatic β-cells. These tissues are key tissues in the pathogenesis of type II diabetes.

AMPK is a heterotrimeric protein, consisting of α, β and γ-subunits. There are two different α-genes, two different β-genes and three different γ-genes.

When the AMP/ATP ratio increases (i.e. low intracellular glucose levels), AMPK is activated. AMP binds to AMPK and activates the protein allosterically. This induces the phosphorylation of Thr 172 within the activation domain of the α-subunit by an upstream kinase, e.g. LKB1 (a tumour suppressor) and calmodulin dependent protein kinase kinase (CaMKK). CaMKK is activated by an increase in intracellular Ca^{2+}-levels, regardless of the AMP/ATP ratio.

When AMPK is activated by AMP, it phosphorylates a number of target proteins, resulting in an enhanced glucose uptake and lipid oxidation, processes that stimulate energy production. It also turns off energy consuming processes such as glucose and lipid production to restore the energy balance. The decreased energy demand and metabolic changes by AMPK are mediated through its regulation on gene expression. AMPK seems to enhance expression of genes essential for skeletal muscle lipid and oxidative metabolism (i.e. hexokinase II and GLUT4) and suppresses the glucose-induced expression of genes associated with lipogenesis (i.e. fatty acid synthase, pyruvate kinase and acetyl-CoA carboxylase). Also, activation of AMPK by adiponectin suppresses genes involved in endogenous glucose production (i.e. glucose-6-phosphatase and phosphoenolpyruvate carboxykinase.

In pancreatic β-cells, activation of AMPK inhibits insulin secretion. This is probably a protective measure to prevent hypoglycaemia during food deprivation [5]. However, genetic knockout of the AMPK subunit AMPK-α2 in animals results in impaired glucose-stimulated insulin secretion [6], indicating a requirement of the enzyme in the β-cell function.

PI3K

PI3K is activated by phosphorylation at low glucose levels. The active enzyme phosphorylates phosphatidyl inositol lipids at the D-3 position on the inositol ring. Due to this, several coordinated processes are set in motion. The final results of these processes are: cell growth,

cell cycle entry, cell migration and cell survival. At high glucose levels, the enzyme is deactivated.

There are several signalling proteins that bind to D3-phosphorylated phosphoinositides, e.g. protein serine-threonine kinases, protein tyrosine kinases and exchange factors that regulates G-proteins. These proteins are normally located in the cytosol, when the cell is unstimulated. But when the phosphoinositides are phoshphorylated, they will accumulate at the plasma membrane by association to the newly activated phosholipid. Possible local responses to phoshoinositide phosphorylation are polymerisation of actin, assembly of signalling complexes and priming of protein kinase cascades. Hyper activation of one or more of the PI3K pathways can lead to cancers and defects in a pathway may cause type 2 diabetes [7].

PI3K activation appears to determine liver, muscle and adipose tissue sensitivity to insulin. In pancreatic b-cells, PI3K acts as a negative regulator on insulin secretion.

Material and methods

The effect of BLX-1002 on AMPK in MIN6 cells was examined with the Western blot method. MIN6 is a mouse insulin-secreting cell line. It is a good model for studying pancreatic β-cells because they share major similarities with the normal β-cells, such as glucose-stimulated insulin secretion. The MIN6 cells were therefore used to test the experimental set-up. When the experimental set-up was confirmed as functional, the MIN6 cells were exchanged for ob/ob mouse islet cells. Mouse islet cells are better models for human islet cells, since MIN6 cells are tumour cells.

The ob/ob mouse islet cells were also used to study the effect of BLX-1002 on PI3K.

MIN6 cells

The MIN6 cells were cultured in DMEM medium containing 25 mM glucose, supplemented with L-glutamine, sodium bicarbonate, 100 IU/ml penicillin, 100 μg/ml streptomycin, 0.5 μl % β-mercaptoethanol/ l medium and 15% fetal bovine serum (FBS, heat inactivated at 56°C for 30 min). The cells were kept in a 37°C incubator with 5% CO_2, 95% air with a moisture atmosphere. Every third day the medium was changed to fresh medium. To detach the cells they were incubated 1-2 minutes with trypsine/EDTA. Before trypsination, the cells were washed with PBS (80mM Na2HPO4, 20mM NaH2PO4 and 100mM NaCl) to wash off the serum.

Before stimulation with BLX-1002, cells from three confluent 75 cm2 culture flasks were harvested by trypsination and collected into a tube. The cells had been serum-starved for ~16 h before the harvest. The cells were resuspended in KRBH buffer (135mM, NaCl, 3.6 mM KCl. 5mM NaHCO3, 0.5mM NaH2PO4, 0.5 MgCl2, 1.5mM CaCl2 and 10mM HEPES) pH 7.4 with 1% followed by washing twice with KRBH buffer with 0.1 % BSA. The cells were then resuspended in KRBH buffer with 0.1% BSA and 3 mM glucose and were incubated for 40 minutes in a 37°C incubator.

At the end of incubation, cells were collected by a brief centrifugation and were resuspended in fresh buffer and divided equally into eight tubes. The cells were now stimulated with BLX-1002 at 37°C. Tube no.1 contained 3mM glucose and was incubated for 5 minutes; tube no.2 contained 20 mM glucose and was incubated for 5 minutes; tube no. 3 contained 20 mM glucose and was incubated for 60 minutes; tube no.4 contained 20 mM glucose and 10 μM BLX-1002 and was incubated for 5 minutes; tube no.5 contained 20 mM glucose and 10 μM BLX-1002 and was incubated for 15 minutes; tube no.6 contained 20 mM glucose and 10 μM BLX-1002 and was incubated for 30 minutes; tube no.7 contained 20 mM glucose and 10 μM BLX-1002 and was incubated for 60 minutes and tube no.8 contained 20mM glucose and 1mM Metformin. Tube 1,2,3 and 8 were used as controls. The activity of AMPK is high at low glucose levels and low at high glucose levels. Metformin is a known activator of AMPK [8].

The incubation was stopped by immediately putting the tubes on ice and the centrifuging them at 5000 × g at 4 °C for 1 minute. The supernatant was discarded.

Ob/ob mouse islet cells

The effects of BLX-1002 on the β-cell function and kinase activity were also investigated in the pancreatic islet cells from *ob/ob* mouse. The islet cells were isolated from pancreases of

four *ob/ob* mice in each experiment (except for the first stimulation where only three mice were applied). Dr. Qimin Zhang performed the isolation of the islets from the pancreas.

The reason for using *ob/ob* mouse is that more than 90% of the pancreatic islet cells in the mouse are β-cells. The *ob/ob* mice came from a colony bred at KI SÖS Stockholm. This colony was established at KI SÖS in 2004 from breeding pairs provided by Professor Janove Sehlin at Umeå University.

The mice were killed by CO2-inhalation, just before the cutting out of the pancreas. The pancreases were put in ice-cold Buffer A (0.1M HEPES, 12.5mM NaCl, 0.5mM KCl, 0.1mM CaCl2 and 0.1mM MgCl2) pH 7.4 with 0.1% BSA. They were then cut into small pieces of equal size that were digested with collagenase (3 mg/ml Buffer A). After washing with Buffer A/0.1 % BSA, DNAse was applied to remove the DNA in the preparation. After washing several times with Buffer A/0.1% BSA, the islets were then separated and picked out under microscope.

The islets were disrupted into single cells by shaking in disruption solution (125 mM NaCl, 5 mM KCl, 1 mM EDTA, 25 mM HEPES and 1 % BSA). The cells were then cultured in RPMI-1640 medium containing 10 % FBS in an incubator overnight.

Before stimulation, the cells were serum-starved for ~16h. The cells were harvested and washed twice in buffer A/0.1% BSA. The cells were then resuspended in buffer A/0.1 %BSA/3 mM glucose, followed by incubation in an incubator for 40 minutes. After the incubation, the cells were resuspended in fresh buffer A/0.1% BSA/3 mM glucose and were divided in equal amounts into 8 tubes.

The cells were now stimulated with BLX-1002 at 37° C. The BLX-1002 used came from BEXEL Pharmaceuticals Inc. Tube no.1 contained 3mM glucose and was incubated for 5 minutes; tube no.2 contained 3 mM glucose and was incubated for 60 minutes; tube no. 3 contained 20 mM glucose and was incubated for 5 minutes; tube no.4 contained 20 mM glucose and was incubated for 60 minutes; tube no.5 contained 20 mM glucose and 10 μM BLX-1002 and was incubated for 5 minutes; tube no.6 contained 20 mM glucose and 10 μM BLX-1002 and was incubated for 15 minutes; tube no.7 contained 20mM glucose and10 μM BLX-1002 and was incubated for 60 minutes and tube no.8 contained 20mM glucose and 1mM Metformin. Tube 1,2,3,4 and 8 were used as controls. The activity of AMPK and PI3K is high at low glucose levels and low at high glucose levels. Metformin is a known activator of AMPK [8]. The incubation was stopped in the same way as for the MIN6 cells.

Western blot

The stimulated cells (MIN6 and *ob/ob* mouse islet cells) were lysed with 60 μl of RIPA (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1mM EDTA and 0.1% SDS) with 1 mM PMSF and proteinase inhibitor cocktail on ice for 30 minutes with occasional vortexing. The tubes were the centrifuged and the supernatant was saved in fresh tubes. 5 μl from each samples was used to measure the protein concentration with Micro BCA^{TM} Protein Assay kit. 20 μl were used for SDS-PAGE and Western blot.

The samples were mixed with SDS-PAGE sample buffer in the presence of 10% β-mercaptoethanol. The volume of the sample buffer was equal to the volume of the samples. The samples were then boiled for 5 minutes at 95^{o} C before being loaded on a 10% SDS-

PAGE. The running buffer (electrode buffer) used for SDS-PAGE contains: 25 mM Tris base, 190 mM glycine and 1% SDS, pH 8.3. The gel was run at 150 V for about 1 h and 15 minutes at room temperature.

The proteins in the gel were transferred to a nitrocellulose membrane at 100 V at RT for 1 h in transfer buffer (25 mM Tris base, 190 mM glycine and 20 % methanol). The membrane was washed in TBS-T buffer (20mM Tris base, 137mM NaCl, 0.025% Tween 20, pH 7.6) and was incubated in blocking solution (TBS-T buffer/5 % fat-free dry milk) over night at 4°C. The blocked membrane was washed with TBS-T buffer and was then incubated with primary antibody in TBS-T buffer with 1 % BSA, 0.02% sodium azide and antibody) at 4° C over night.

At the end of the incubation, the membrane was washed extensively with TBS-T buffer (two quick washes followed by four 5-10 minute washings). It was then incubated in the secondary antibody in TBS-T buffer, 1% BSA and antibody for 1 h at RT with shaking. After incubation, the membrane was washed extensively.

The secondary antibody was coupled to horseradish peroxidase. By soaking the membrane in Western Blotting Luminol Reagent (sc-2048 from Santa Cruz Biotechnology Inc.), it could be used to expose X-ray films. The band density was measured with a computer program Quantity One.

Each membrane containing samples from MIN6 was probed with two different antibodies: phospho-AMPKα (Thr172) antibody (#2531 from Cell Signalling Technology®) and monoclonal anti-β-actin (clone AC-15 product no. A1978 from SIGMA®). Anti-β-actin is used to estimate the total amount of protein loaded on the gel. The membranes containing *ob/ob* mouse islet cell samples were all probed with three different antibodies: phospho-AMPKα (Thr172) antibody, phospho-PI3K p85α (Tyr 508) and monoclonal anti-β-actin. The dilution used for all primary antibodies was 1:1000 except for anti-β-actin where a dilution of 1:2400 was used.

The secondary antibody used for P_i-AMPK was goat anti-rabbit IgG-HRP (sc-2004 from Santa Cruz Biotechnology Inc). The secondary antibody used for P_i-PI3K was donkey anti-goat IgG-HRP (sc-2020 from Santa Cruz Biotechnology inc) and for β-actin, goat anti-mouse IgG_1-HRP (sc-2060 from Santa Cruz Biotechnology Inc). The dilution used for all secondary antibodies were 1:10 000.

Insulin secretion study

Since the Western blot experiment indicated that BLX-1002 activates the two enzymes, a follow up study on BLX-1002 induced insulin secretion was performed. The effect of BLX-1002 on insulin secretion was studied in the presence of inhibitors of the two enzymes. The inhibitors used were Ly294002 (Calbiochem®) and wortmannin (Sigma®), inhibitors for PI3K, and compound C (Calbiochem®), an inhibitor for AMPK.

Ob/ob mouse islet cells were once again used, but this time they were not starved over night before the experiment. Otherwise, the cells were harvested and cultured in the same way as for the Western blot experiments.

Before the experiment, the cells were washed two times in Buffer A/0.1% BSA and once in Buffer A/0.1% BSA/3mM glucose. The cells were then resuspended in 9 ml of Buffer A/0.1% BSA/3 mM glucose and were divided equally into 9 tubes. The inhibitors wortmannin or compound C were added to their respective tubes and the same amount of DMSO was added to the control tubes. The cells were now incubated on a shaker in a 37°C incubator for 15 minutes. Now the Ly294002 inhibitor was added to the remaining tubes. All tubes were incubated for another 15 minutes in the incubator.

After the pre-incubation with the inhibitors, BLX-1002 were added in the corresponding tubes and then glucose was added to a final concentration of 20 mM in all tubes except one. The samples were immediately transferred to a 48-well plate. All samples were divided into 3 different wells. The incubation immediately started by adding glucose and placing the plate in a 37°C incubator and it lasted for 20 minutes. The reaction was stopped by immediately transferring the plate to ice after the incubation. The content of the wells was transferred to ice-cold tubes and the tubes were centrifuged at 5000 × g, 4° C for 1 minute. The supernatant was transferred to new, ice-cold tubes. The samples were either saved in a -70°C freezer or used directly to measure the insulin content with a Mercodia Mouse Insulin ELISA kit.

The incubation conditions are as follows: sample 1 had 3 mM glucose and DMSO, sample 2 had 20 mM glucose and DMSO; sample 3 had 20 mM glucose; DMSO and 10 μM BLX-1002; sample 4 had 20 mM glucose and compound C; sample 5 had 20 mM glucose, compound C and BLX-1002; sample 6 had 20 mM glucose and wortmannin; sample 7 had 20 mM glucose, wortmannin and 10 μM BLX-1002; sample 8 had 20 mM glucose and Ly294002 and sample 9 had 20mM glucose, Ly294002 and 10 μM BLX-1002.

Results

Bi-phasic effects of BLX-1002 on AMPK and PI3K activation

When the relative values of the β-actin measurements were compared with those of the protein assay, large differences were found. There should not be any big differences between the two methods of measuring protein amount, since the same amount of every sample was loaded on the SDS-PAGE gel. This led to a suspicion that the density measurements of β-actin bands were incorrect. The calculations were then performed with the values from the protein assay instead of the values from the β-actin measurements.

Western blot - MIN6

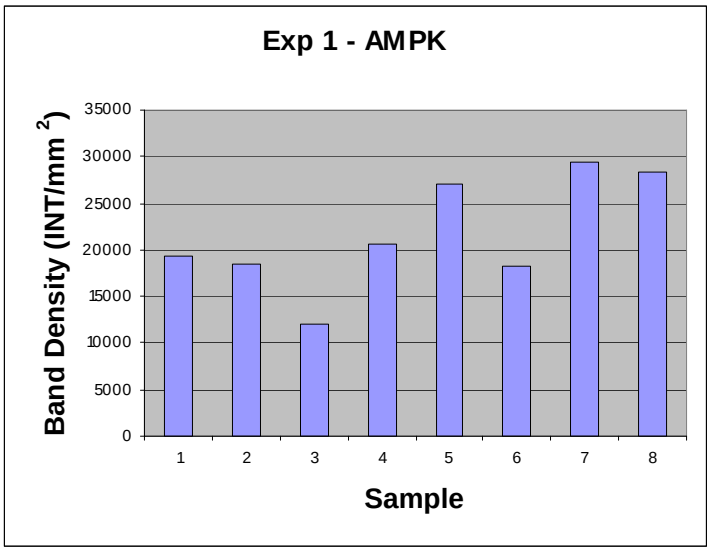


Fig. 2. This is the bar chart from the first repetition of the MIN6 experiment (AMPK). Sample 1 represents low glucose and 5 minutes incubation; sample 2 is high glucose and 5 minutes incubation; sample 3 is high glucose and 60 minutes incubation, sample 4 is high glucose with BLX-1002 present and 5 minutes incubation; sample 5 is high glucose with BLX-1002 present and 15 minutes incubation, sample 6 is high glucose with BLX-1002 present and 30 minutes incubation, sample 7 is high glucose with BLX-1002 present and 60 minutes incubation and sample 8 is high glucose with metformin present and 60 minutes incubation. The data is normalised with the results from the protein assay.

Figure 2 represents the first repetition of the MIN6 experiment. The 5 minutes low glucose control has only a little more AMPK activity than the 5 minutes high glucose control. The 60 minutes high glucose control is much lower, but there is no 60 minutes low glucose control to compare it to. The metformin control has a much higher activity than the 60 minutes high glucose control.

The 5 and 15 minutes BLX-1002 samples have a higher amount of activated AMPK than the 5 minutes high glucose control. The 30 minutes BLX-1002 sample has a higher activity of AMPK than the 5 minutes high glucose control, but slightly less activity than the 60 minutes high glucose control. The 60 minutes BLX-1002 sample has a higher activity than the 60 minutes high glucose control.

Western blot - ob/ob mouse islet cells

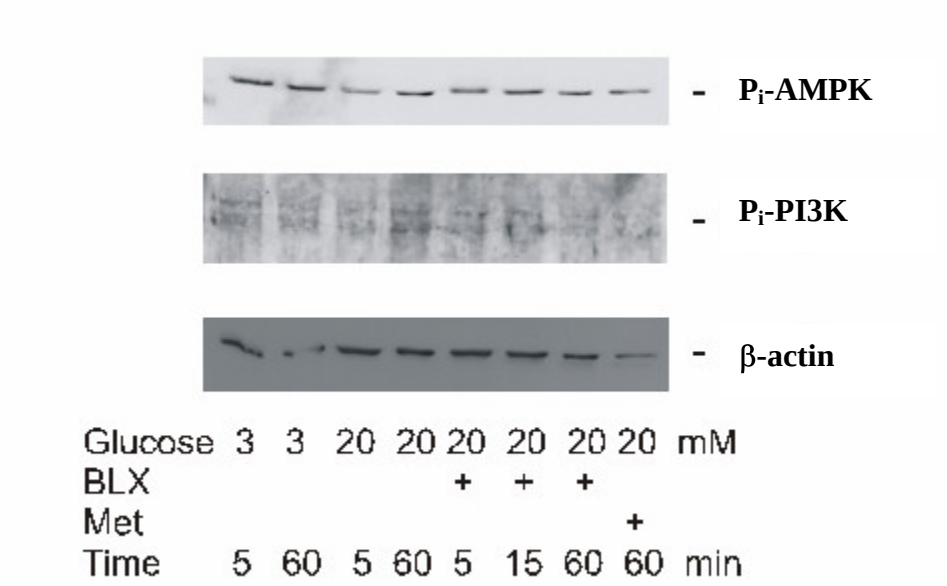
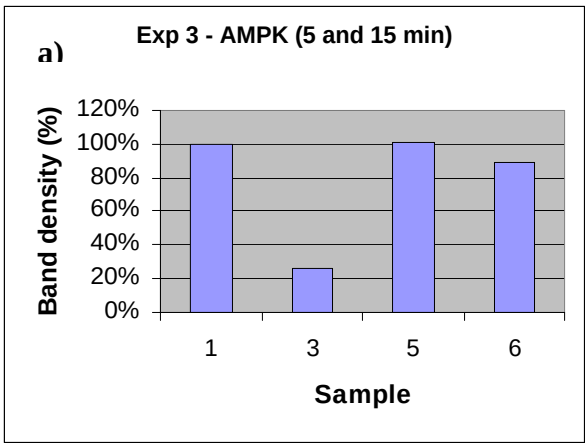
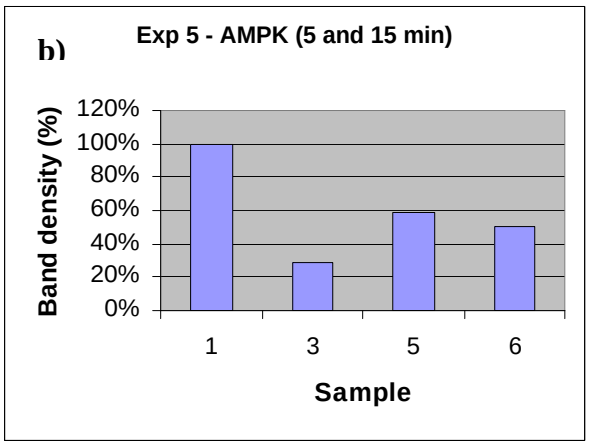


Fig. 3. These are pictures of an exposed membrane from the experiment on ob/ob mouse. Picture a shows the exposure for P_i-AMPK, picture b represents P_i-PI3K and picture c represents β-actin (overall protein amount).

Figure 3 shows the exposures of one of the Western blot membranes from the experiments on ob/ob mouse islet cells. The exposure of β-actin shows the overall protein amount in each sample. It is clear from the picture that the total amount of protein is different in the different samples. That makes it difficult to make any conclusions on the difference in AMPK and PI3K activity in the different samples, just by looking at the exposures. The bar charts where the results have been normalised by the total protein amount will be easier to use.

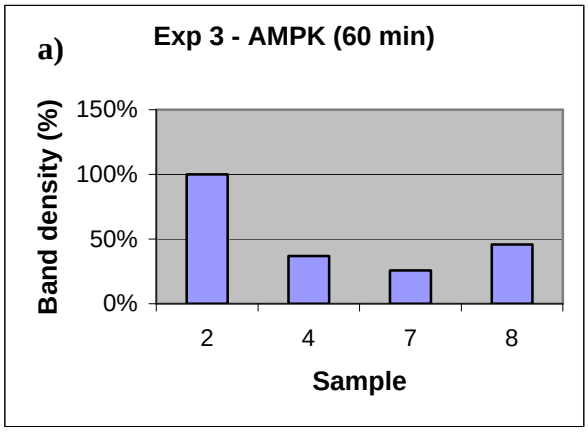
AMPK

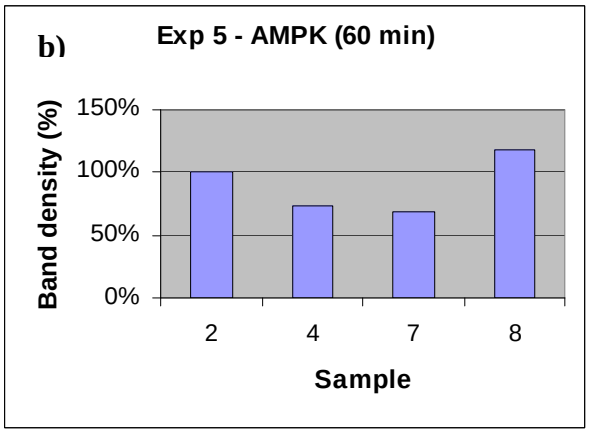




Glucose	**3**	**20**	**20**	**20**	**3**	**20**	**20**	**20**	**mM**
BLX-1002	**-**	**-**	**+**	**+**	**-**	**-**	**+**	**+**	
Time	**5**	**5**	**5**	**15**	**5**	**5**	**5**	**15**	**min**

*Fig.4 a-b. The bar charts above represent all the samples with an incubation time of 5 and 15 minutes from the Western blot experiment on AMPK. **Sample 1** is set as **100 %**. The data are normalised with the results from the protein assay.*



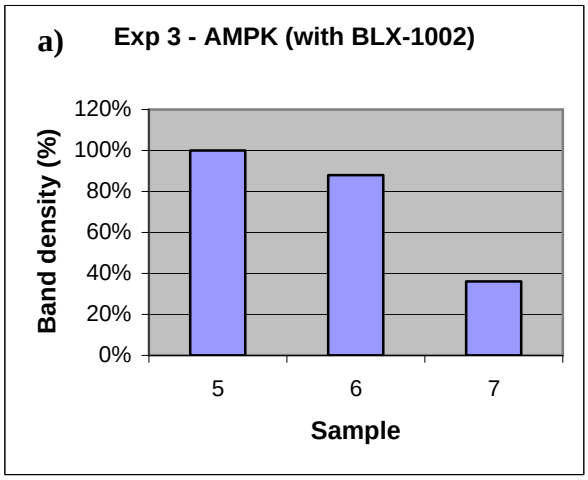


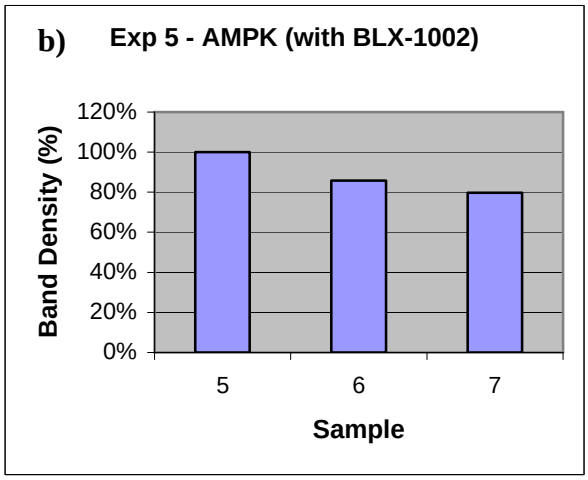
Glucose	**3**	**20**	**20**	**20**	**3**	**20**	**20**	**20 mM**
BLX-1002	-	-	+	-	-	-	+	-
Metform.	-	-	-	+	-	-	-	+

*Fig. 5 a-b. The bar charts above represent all the samples with an incubation time of 60 minutes from the Western blot experiment on AMPK. **Sample 2** is set as **100 %**. The data are normalised with the results from the protein assay.*

In the western blot experiment on AMPK, the controls indicate that the results are trustworthy. In both experiments, high glucose levels (20 mM) inhibit AMPK activity at 5 and 60 minutes incubation and incubation with metformin activates AMPK at 20 mM glucose (fig 4-5). This means that the cells have responded to the stimulation.

The data shows activation of AMPK by BLX-1002 after both 5 and 15 minutes incubation (fig. 4), but not after 60 minutes incubation (fig. 5). After 60 minutes incubation with BLX-1002, the drug induced a slight inhibition of AMPK.



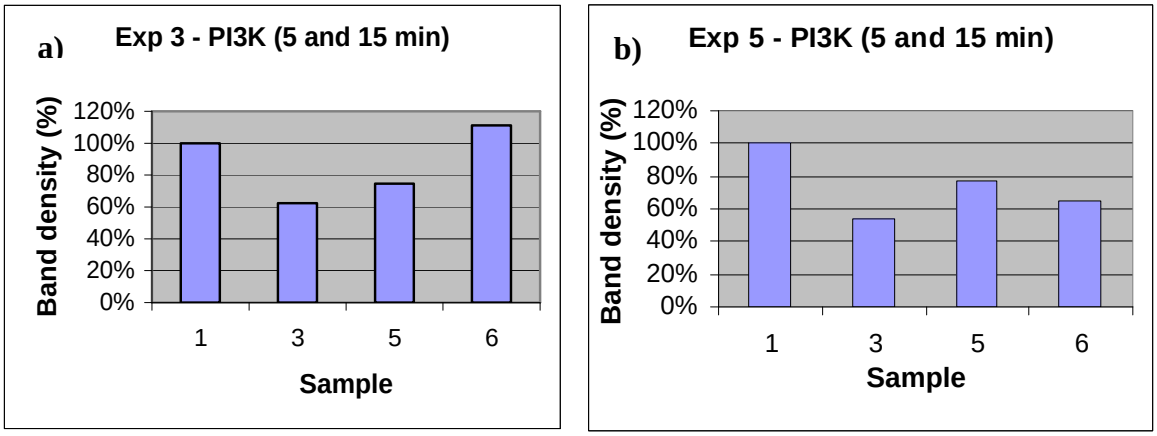


Time	**5**	**15**	**60**	**5**	**15**	**60**	**min**

*Fig. 6 a-b. The bar charts above represent all the samples from the Western blot experiment on AMPK that was incubated with 10 μM BLX-1002 (at 20 mM glucose). **Sample 5** is set as **100 %**. The data are normalised with the results from the protein assay.*

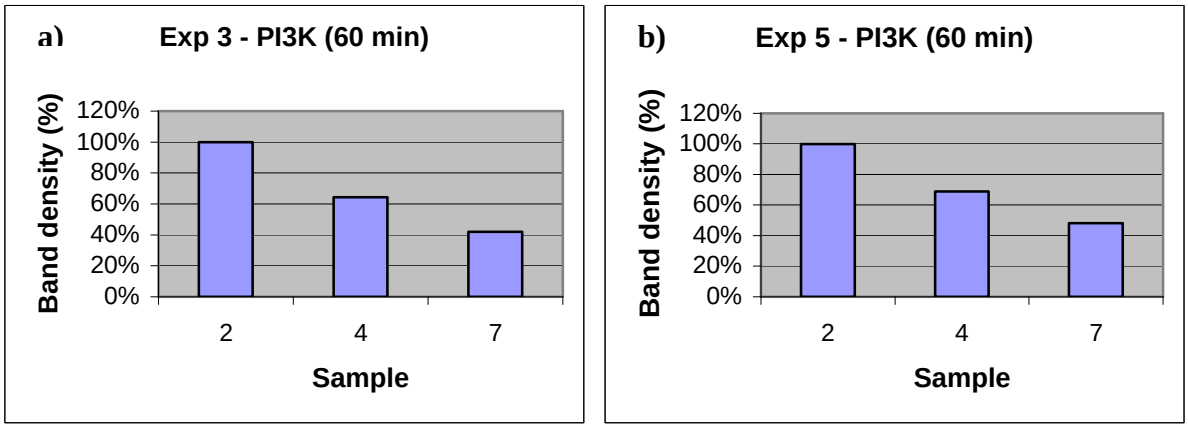
The data indicates that the BLX-1002 mediated AMPK activation lasts for at least 15 minutes, but after 60 minutes the activity of AMPK has declined (fig 6).

PI3K



Glucose	**3**	**20**	**20**	**20**	**3**	**20**	**20**	**20**	**mM**
BLX-1002	**-**	**-**	**+**	**+**	**-**	**-**	**+**	**+**	
Time	**5**	**5**	**5**	**15**	**5**	**5**	**5**	**15**	**min**

*Fig. 7 a-b. The bar charts above represent all the samples with an incubation time of 5 or 15 minutes from the Western blot experiment on PI3K. **Sample 1** is set as **100 %**. The data are normalised with the results from the protein assay.*

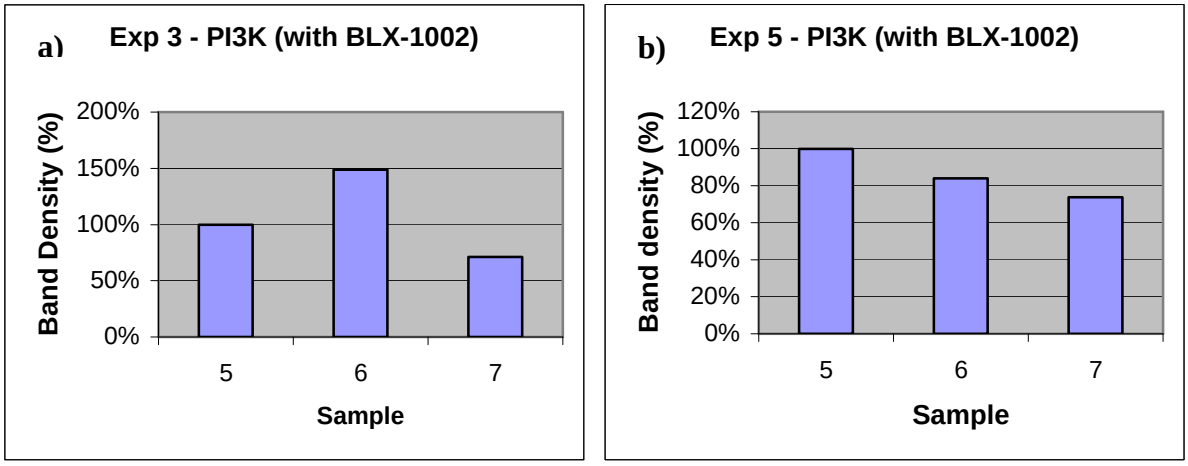


Glucose	**3**	**20**	**20**	**3**	**20**	**20**	**mM**
BLX-1002	**-**	**-**	**+**	**-**	**-**	**+**	

*Fig. 8 a-b. The bar charts above represent all the samples with an incubation time of 60 minutes from the Western blot experiment on PI3K. **Sample 2** is set as **100 %**. The data are normalised with the results from the protein assay.*

The results from the Western blot experiment on PI3K looks trustworthy as well. In both bar charts, high glucose concentration (20 mM) has inhibited PI3K, both at 5 and 60 minutes incubation (fig. 7-8). Metformin cannot be used as a control for PI3K, since it is not an activator of the enzyme.

According to the bar charts in figure 7, PI3K is activated after 5 minutes incubation with BLX-1002. But after 60 minutes incubation with BLX-1002 PI3K activity seems to have declined compared to incubation without BLX-1002 (fig 8).

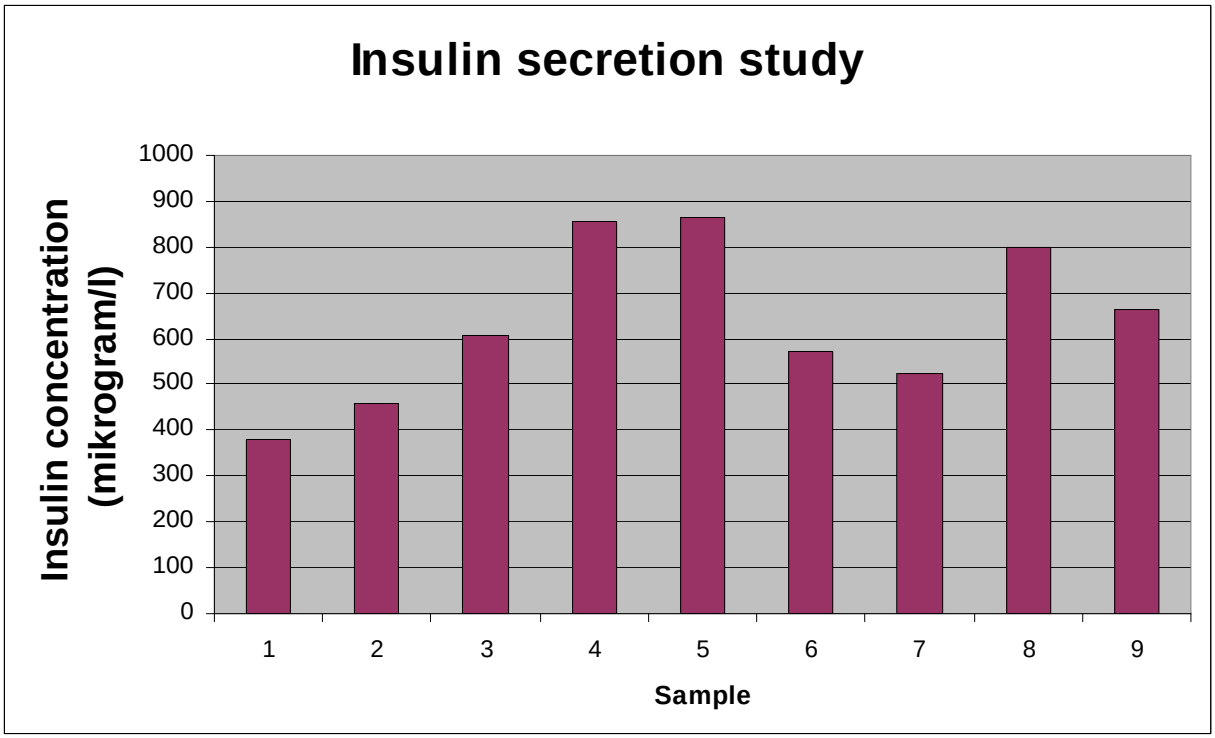


Time	5	15	60	5	15	60	min

Fig. 9 a-b. The bar charts above represent all the samples from the Western blot experiment on PI3K that was incubated with 10 μM BLX-1002 (at 20 mM glucose). ***Sample 5*** *is set as* ***100 %****. The data are normalised with the results from the protein assay.*

Figure 9 indicates that the activation of PI3K by BLX-1002 is indeed a short time response. After 15 minutes incubation with the compound, PI3K activity declines.

Involvement of AMP-activated Protein Kinase and Phosphoinositide-3-kinase in BLX-1002 induced insulin secretion



Glucose	3	20	20	20	20	20	20	20	20 mM
BLX-1002	-	-	+	-	+	-	+	-	+
Inhibitor	-	-	-	Cmp C	Cmp C	Wort.	Wort	LY.	LY.
Inhibited enzyme	-	-	-	AMPK	AMPK	PI3K	PI3K	PI3K	PI3K

Fig.10. This bar chart represents the results from the insulin secretion study. The chart represents a triplicate experiment. All samples were incubated for 20 minutes at 37 ℃. Cmp C is short for Compound C, Wort. Is short for Wortmannin and LY. Is short for LY294002.

The results from the insulin secretion study (fig. 10) seem trustworthy, although only one experiment was performed. The addition of glucose (final concentration 20 mM) increases the secretion of insulin, compared to the secretion at 3 mM glucose, although the increase is smaller than expected. Addition of BLX-1002 induced an enhanced insulin secretion, compared to 20 mM glucose alone.

According to figure 10, addition of the AMPK inhibitor Compound C at 20 mM glucose further stimulates the secretion of insulin. This is consistent with the fact that AMPK inhibits glucose induced insulin secretion [5]. The same thing happens when either of the two PI3K inhibitors, Wortmannin and LY294002, is added at 20 mM glucose. This is consistent with the fact that PI3K inhibits glucose induced insulin secretion.

After pre-incubation with the AMPK inhibitor Compound C at 20 mM glucose, the BLX-1002 induced insulin secretion is blocked. The same thing happens when BLX-1002 is added after pre-incubation with either one of the PI3K inhibitors, LY294002 and Wortmannin, at 20 mM glucose. There is even a slight inhibition in insulin secretion when BLX-1002 is added after inhibiting PI3K.

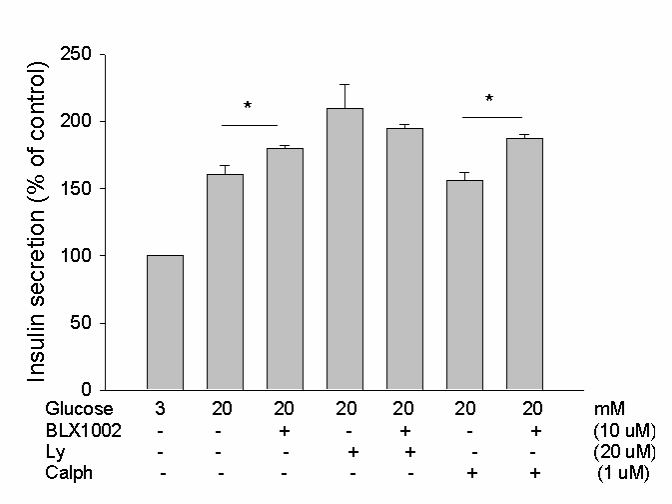


Fig. 11. The bar chart represents a preliminary result from the Diabetes group at KISÖS in Stockholm, where insulin secretion from ob/ob mouse β-cells have been measured. Cells were pre-incubated in the presence of LY29400 (a specific PI3K inhibitor, 20 mM), Calphostin C (a specific PKC inhibitor, 1 mM) or vehicle for 15 min, followed by addition of BLX-1002. Incubation was initiated by addition of glucose and lasted for 20 min, at 37 ^{o}C. A triplicate experiment is shown (P < 0.05 by ANOVA).*

For LY294002, similar results (fig.11) were obtained in a previous experiment performed by the Diabetes group at KI SÖS, Stockholm. This experiment also showed that pre-incubation with LY294002 blocks BLX-1002 induced insulin secretion.

Discussion

MIN6 cells

The data from the MIN6 experiment seems somewhat trustworthy. The metformin control seems to be working fine and at 60 minutes, high glucose seems to inhibit AMPK. The problem is that there is no 60 minutes low glucose control to compare the 60 minutes high glucose control to. BLX-1002 seems to activate AMPK after 5 and 60 minutes. In this sole experiment, the activity increased between 5 and 15 minutes. It then went down after 30 minutes, only to go up again at 60 minutes incubation.

No real conclusion of the effect of BLX-1002 on AMPK can be drawn from an experiment on tumour cells. But the experiment showed that the experimental set up worked well enough to start working on *ob/ob* mouse islet cells.

ob/ob mouse islet cells

AMPK

AMPK is stimulated by low glucose concentrations [5] and metformin [8]. Therefore, the activity of the enzyme (amount of P_i-AMPK) is expected to be higher in the low glucose controls and in the metformin control than in the high glucose control. Since the controls behaved as expected, conclusions can be drawn from activity level of AMPK in the BLX-1002 samples.

BLX-1002 induced temporary activation of AMPK in the pancreatic β-cells. It tends to stimulate AMPK during 5-15 min incubation but somewhere between 15-and 60 minutes the activation of AMPK by BLX-1002 starts to decline. There may even be a slight inhibition of the enzyme by BLX-1002 after 60 minutes.

The insulin secretion study indicated that AMPK is necessary for BLX-1002 induced insulin secretion. When the cells were pre-incubated with the AMPK inhibitor Compound C, the effect of BLX-1002 on insulin secretion was blocked.

It is a bit contradictory that BLX-1002 would activate AMPK, an enzyme that inhibits insulin secretion when it is active, since BLX-1002 increases insulin secretion after 20 minutes incubation. But as mentioned in the introduction, genetic knockout of the subunit AMPK-α2 in animal models leads to impaired insulin secretion [6] and indicates that AMPK may in fact be required for β-cell function. These findings suggest that the role of AMPK on insulin secretion may depend upon different signalling pathways initiated by different insulin secretory modulators Further research on the role of AMPK in insulin secretion has to be performed in order to confirm the results presented in this report.

PI3K

In the case of PI3K, BLX-1002 induced a temporary activation of the enzyme. The activation has a tendency to last at least 15 minutes after the start of the incubation. After 60 minutes, the activation of the enzyme is replaced by an inhibition.

According to the insulin secretion study, inhibition of PI3K blocks the BLX-1002 induced insulin secretion. This indicates that PI3K is also required in BLX-1002 induced insulin

secretion. There is evidence that both denies and supports this result. PI3K has been shown to suppress glucose stimulated insulin secretion by affecting the levels of intracellular Ca^{2+} [9]. Also, PI3K inhibitors have been shown to increase insulin secretion in 832/13 rat insulinoma cells [10].

One piece of evidence for a requirement of PI3K in insulin secretion is that in mice lacking the p110gamma isoform of PI3K, glucose stimulated insulin secretion was impaired [11]. Also, L-783,281 evokes intracellular [Ca^{2+}] increases and Ca^{2+}-exocytosis in β-cells via an IRS-1/PI3K-dependent pathway. The increase in intracellular [Ca^{2+}] involves release of Ca^{2+} from intracellular stores [12]. Ca^{2+} is involved in glucose stimulated insulin release. One further occasion where PI3K is involved in insulin secretion is when leptin constrains phospholipase C-protein kinase C-induced insulin secretion. The effect of leptin is mediated through a PI3K-dependent pathway [13]. The above findings suggest that the role of PI3K on insulin secretion may depend upon different signalling pathways initiated by different insulin secretory modulators. To explain the role of PI3K on BLX-1002-induced insulin secretion, further study has to be performed on the subject.

Conclusion

Figure 12 shows the proposed connections between BLX-1002 and the two enzymes AMPK and PI3K. Also included is the already established connection between BLX-1002 and insulin secretion as well as the connections between glucose, AMPK, PI3K and insulin secretion. Glucose and BLX-1002 stimulates insulin secretion. AMPK and PI3K, on the other hand, inhibit insulin secretion. The two enzymes, in their turn, are inhibited when the intracellular glucose concentration is high.

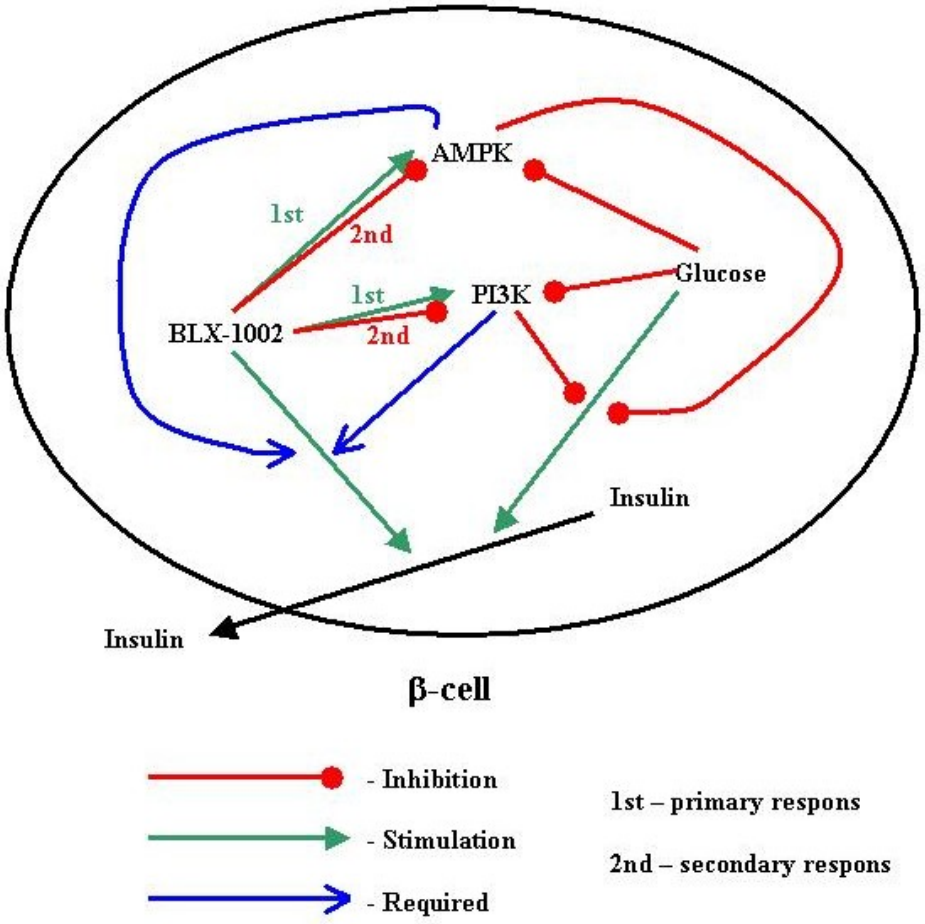


Fig. 12. This figure shows the connections between AMPK, PI3K, BLX-1002, glucose and insulin secretion within a β-cell. The proposed connection between BLX-1002 and the two enzymes are based on the results presented in this report.

BLX-1002 activates both AMPK and PI3K. In both cases, the activation is temporary and seems to be followed by an inhibition. Although both enzymes inhibit glucose induced insulin secretion, they seem to be required for BLX-1002 induced insulin secretion. When the enzymes were inhibited, the effect of BLX-1002 on insulin secretion was blocked. To explain and confirm these findings, more research has to be performed on the subject.

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