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Mechanism of pure glucose-dependent insulinotropic activity of a novel imidazoline compound BL11282

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To my family

ABSTRACT

We developed a novel, pure glucose-dependent, insulinotropic imidazoline compound, BL11282, which directly affects the insulin exocytotic machinery and does not block the K_{ATP} channel activity. BL11282 does not induce insulin secretion at basal glucose concentrations, whereas it stimulates insulin secretion at an elevated glucose level. Therefore this imidazoline should not provoke hypoglycemic episodes as it has been observed for the sulfonylureas. However, so far, the detailed biochemical and pharmacological mechanisms underlying the insulinotropic effects of BL11282 are not fully established. The overall objective of this study was to investigate signal-transduction pathways involved in the pure glucose-dependent activity of BL11282 on insulin release. Using $SUR1^{(-/-)}$ mice, we unambiguously confirmed the previous notion that the insulinotropic activity of BL11282 is unrelated to its interaction with ATP-dependent K^+ channels. We have also shown that previously described targets for imidazoline compounds like α_2-adrenoreceptors, imidazoline I_1-receptors and monomeric G-protein Rhes are not involved in the mechanisms of the insulinotropic action of BL11282. To clarify the molecular mechanisms underlying the effects of BL11282 on insulin secretion, we have used an approach involving desensitization of ß-cells to the insulinotropic activity of BL11282 by prolonged incubation with the compound. The data obtained show that overnight pretreatment of pancreatic islets with BL11282 desensitizes the subsequent islet response to this imidazoline. Islets pretreated for the same time with efaroxan, another insulinotropic imidazoline, are unresponsive to subsequent addition of efaroxan but preserve their response to BL11282. The effect of pretreatment with BL11282 is accompanied by an increased insulinotropic response to subsequent high glucose concentration. Desensitization of islet response to BL11282 does not eliminate subsequent islet response to GLP-1, but significantly decreases the fold potentiation of insulin release by this peptide. The latter effect points to the importance of GLP-1 stimulated signal-transduction pathways for the insulinotropic activity of BL11282. Our results support the involvement of the cAMP-GEFII·Rim2 pathway in BL11282 stimulated insulin secretion. Indeed, expression of dominant negative cAMP-GEFII and Rim2 mutant proteins in MIN6 cells lead to a significant reduction in insulin secretion stimulated by the imidazoline. To further investigate this direct mechanism of BL11282 on insulin release, we turned our attention to calcium-independent PLA_2 isoform $iPLA_2\beta$, which is predominantly expressed in pancreatic islets and plays an important role in insulin secretion in pancreatic islets. Our observations indicate a deficiency in $iPLA_2\beta$ isoform expression in diabetic Goto-Kakizaki (GK) rat islets compared to Wistar rat islets, this effect being in agreement with an impairment in the glucose-stimulated insulin response in GK rat islets. Pharmacological inhibition of $iPLA_2$ and cytochrome P-450 enzymes completely abolished the insulinotropic effect of BL11282. BL11282 stimulated arachidonic acid release from the islets in the presence of high glucose concentration and this effect was fully blocked by $iPLA_2$ inhibitor, bromoenol lactone. The data suggest that potentiation of glucose-induced insulin release by BL11282, independent of concomitant changes in cytoplasmic free Ca^{2+} concentration, involves the release of arachidonic acid by $iPLA_2$ and its metabolism to epoxyeicosatrienoic acids through the cytochrome P-450 pathway.

Key words: insulin secretion; pancreatic islets; imidazolines; BL11282; arachidonic acid; phospholipase; cytochrome P-450; GLP-1; desensitization; Rhes

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LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I. **Vladimir V. Sharoyko,** Barbara Leibiger, Irina I. Zaitseva, Suad Efendić, Per-Olof Berggren and Sergei V. Zaitsev. The imidazoline compound BL11282 stimulates insulin release through the cAMP-GEFII·Rim2 pathway. *Submitted*

II. **Vladimir V. Sharoyko**, Irina I. Zaitseva, Barbara Leibiger, Suad Efendić, Per-Olof Berggren and Sergei V. Zaitsev. Arachidonic acid signaling is involved in the mechanism of imidazoline-induced K_{ATP} channel-independent stimulation of insulin secretion. *Cellular and Molecular Life Sciences*. 64: 2985-2993, 2007

III. **Vladimir V. Sharoyko**, Irina I. Zaitseva, Mark Varsanyi, Neil Portwood, Barbara Leibiger, Ingo Leibiger, Per-Olof Berggren, Suad Efendić and Sergei V. Zaitsev. Monomeric G-protein, Rhes, is not an imidazoline-regulated protein in pancreatic β-cells. *Biochem Biophys Res Commun.* 338(3): 1455-1459, 2007

OTHER PUBLICATIONS BY THE SAME AUTHOR

I. Theres Jägerbrink, Helena Lexander, Carina Palmberg, Jawed Shafqat, **Vladimir V. Sharoyko**, Per-Olof Berggren, Suad Efendić, Sergei V. Zaitsev and Hans Jörnvall. Differential protein expression in pancreatic islets after treatment with an imidazoline compound. *Cellular and Molecular Life Sciences*. 64(10): 1310-6, 2007

II. Irina I. Zaitseva, **Vladimir V. Sharoyko**, Joachim Størling, Suad Efendić, Christopher Guerin, Thomas Mandrup-Poulsen, Pierluigi Nicotera, Per-Olof Berggren and Sergei V. Zaitsev. RX871024 reduces NO production but does not protect against pancreatic beta-cell death induced by proinflammatory cytokines. *Biochem Biophys Res Commun.* 347(4): 1121-8, 2006

CONTENTS

LIST OF ABBREVIATIONS

AA	arachidonic acid
AC	adenylate cyclase
ADP	adenosine 5′-diphosphate
ATP	adenosine 5′-triphosphate
BL11282	5-chloro-3-(4,5-dihydro-1H-imidazol-2-yl)-2-methylindole•HCl
BSA	bovine serum albumin
$[Ca^{2+}]_i$	cytoplasmic free Ca^{2+} concentration
$cPLA_2$	cytosolic Ca^{2+}-dependent phospholipase A_2
cAMP	adenosine 3′,5′-cyclic monophosphate
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
EBSS	Earle's balanced salt solution
EEA	epoxyeicosatrienoic acid
EGFP	enhanced green fluorescent protein
EDTA	ethylenediaminetetraacetic acid
FCS	fetal calf serum
GEFII	cAMP-regulated guanine nucleotide exchange factor II
GK rat	Goto-Kakizaki rat
GLP-1	glucagon like peptide-1
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
$iPLA_2$	cytosolic Ca^{2+}-independent phospholipase A_2
K_{ATP} channel	ATP-dependent potassium channel
KRBB	Krebs–Ringer bicarbonate buffer
mRNA	messenger ribonucleic acid
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
PBS	phosphate-buffered saline
PKA	protein kinase A
PKC	protein kinase C
PC-PLC	phosphatidylcholine-specific phospholipase C
PI-PLC	phosphatidylinositol-specific phospholipase C
P450	cytochrome P-450
Rim2	Rab3 interacting molecule
rPL30	ribosomal protein L30
RT-PCR	reverse-transcription polymerase chain reaction
SUR	sulphonylurea receptor (subunit of potassium channel)

1 INTRODUCTION

Treatment of type 2 diabetes is still a difficult task due to the complex nature of the disease (1). Drugs improving the action of insulin on its target tissues and pharmacological secretagogues correcting the deficient insulin secretion by pancreatic β-cells are both used to improve the quality of care of diabetic patients. Unfortunately, until now, blockers of the K_{ATP} channel, sulphonylurea compounds, are the main compounds widely used in the clinical management of type 2 diabetes with deficiency in insulin secretion. However, the effect of these compounds at low glucose concentration leads to an increased risk of hypoglycemia (2). In contrast to sulphonylurea compounds, the peptide GLP-1 and its analogues increase insulin secretion in a pure glucose-dependent manner leading to decreased glucose concentration without the risk of hypoglycemic episodes (3). GLP-1 was shown to be effective in maintaining normoglycemia in diabetic patients (4). The short half-life of the peptide *in vivo* and the requirement for intravenous infusion restricts its application in clinical practice. We have developed an oral insulinotropic imidazoline compound BL11282 (5-chloro-3-(4,5-dihydro-1H-imidazol-2-yl)-2-methylindole hydrochloride) devoid of these disadvantages. BL11282 possesses a pure glucose-dependent insulinotropic activity similar to GLP-1, but, unlike the sulphonylureas, does not affect K_{ATP} channels. In pancreatic islets from spontaneously diabetic GK rats, BL11282 restored the impaired insulin response to glucose (5). This novel, second generation, insulinotropic compound opens fascinating perspectives for the development of new antidiabetic drugs. However, so far, the detailed biochemical and pharmacological mechanisms underlying the K_{ATP} channel-independent effects of BL11282 on insulin release remain unknown. Clarification of these mechanisms should provide valuable knowledge in understanding the disturbances of insulin release in type 2 diabetes, and thereby facilitate our ability to treat this complex disease.

1.1. Mechanisms of insulin secretion in pancreatic β-cells

At least two principal signaling pathways are involved in the stimulation of insulin secretion in pancreatic β-cells (Fig. 1). These are the K_{ATP} channel-dependent and the K_{ATP} channel-independent pathways. In the K_{ATP} channel-dependent pathway, ATP derived from the glycolytic metabolism of glucose closes K_{ATP} channels and

causes β-cell membrane depolarization. The resulting opening of voltage-dependent L-type Ca^{2+} channels increases influx of extracellular Ca^{2+} into the β-cells, leading to rise in $[Ca^{2+}]_i$ which triggers insulin release (6; 7). The existence of the K_{ATP} channel-independent mechanism of stimulation of insulin release was shown over fifteen years ago (8-10). Molecular mechanisms underlying the K_{ATP} channel-dependent stimulation of insulin secretion are well studied (11-13), whereas the mechanisms involved the K_{ATP} channel-independent pathways are still not defined. Nevertheless, several candidate signalling pathways exist.

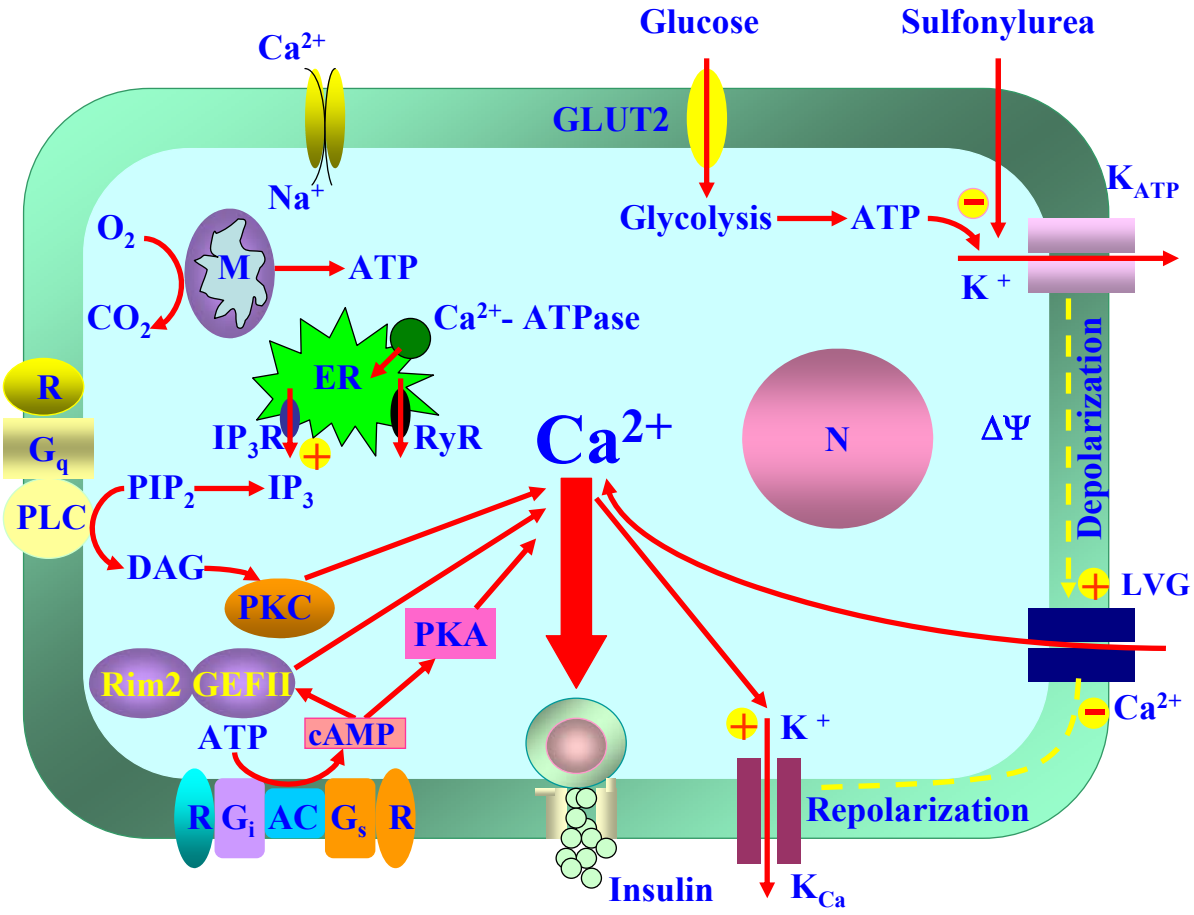

Fig. 1. Schematic model of the signal transduction pathways involved in the insulin secretion process in the pancreatic β-cells.

AC, adenylate cyclase; ATP, adenosine triphosphate; DAG, diacylglycerol; GEFII, cAMP-regulated guanine nucleotide exchange factor II; IP_3, inositol 1,4,5-triphosphate; cAMP, adenosine 3′,5′-cyclic monophosphate; ER, endoplasmic reticulum; K_{ATP}, ATP-dependent potassium channel; K_{Ca}, Ca^{2+}-dependent potassium channel; LVG, voltage-gated L-type channel; M, mitochondria; PIP_2, phosphatidyl inositol 4,5-diphosphate; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; R, receptor; Rim2, Rab3 interacting molecule

Mitochondria-generated signaling. Nutrient metabolism in the β-cell mitochondria generates the messenger molecules which may be involved in K_{ATP} channel-independent mechanism of glucose-stimulated insulin secretion (14; 15). In the pancreatic islet β-cell, mitochondria actively takes up pyruvate, and >90% of glucose-derived pyruvate enters the mitochondria (16). Further metabolism of pyruvate in the mitochondria generates second messengers involved in initiating and maintaining insulin secretion. Glucose, via anaplerosis, induces a rise in the concentration of mitochondrial citrate, which is exported to the cytosol and cleaved to acetyl-CoA and oxaloacetate. Acetyl-CoA is carboxylated to malonyl-CoA. Malonyl-CoA-induced inhibition of CPT-1 suppresses the flux of fatty acids through β-oxidation, with concomitant elevations in the cytosolic content of long-chain acyl-CoAs (17). The long-chain acyl-CoAs have the capacity to act directly (18) or indirectly as signal molecules, e.g., by activating PKC isoforms that can stimulate insulin exocytosis, or by causing the palmitoylation or otherwise acylating, of G proteins (19-21), synaptotagmin (22), SNAP25 (23) and other β-cell exocytotic proteins (24-26). The malonyl-CoA hypothesis is currently controversial (27; 28).

ATP, NADPH and NADH generated by mitochondria are also known to be regulators of the K_{ATP} channel-independent pathway (29; 30). Glucose induces an increase in synthesis of glutamate in the mitochondria, which is exported to the cytosolic compartment where it sensitizes the exocytotic machinery to Ca^{2+} (15; 31).

Adenylate cyclase and PKA signaling. These pathways are inhibited by adrenaline and are activated by vasoactive intestinal peptide, pituitary adenylate cyclase activating peptide, GLP-1, and GIP. These peptides, acting via G_s, stimulate adenylate cyclase and cause a rise in cAMP and the activation of PKA. The increased activity of PKA potentiates insulin secretion (32). GLP-1 possesses a pure glucose-dependent insulinotropic activity (33; 34). GLP-1 can modulate the K_{ATP} channel-independent insulin secretion by PKA-dependent (35) and PKA-independent mechanisms involving a cAMP-binding protein cAMP-GEFII (36-38). cAMP-GEFII was shown to activate mitochondrial ATP-production (39). By interaction with Rim2, Rab3 interacting molecule, cAMP-GEFII mediates cAMP-dependent, PKA-independent exocytosis in GLP-1-potentiated insulin secretion in pancreatic β-cells (37).

PLA_2s and arachidonic acid (AA) signaling. AA and its metabolites may be important mediators in the regulation of the insulin secretion process in islet β-cells. AA is the substrate for the synthesis of eicosanoid signaling molecules (40). In rodent

islets, AA is a major acyl component of glycerolipids, constituting >30% of glycerolipid fatty acid residues (41). AA release depends on ß-cell metabolism of glucose (42). Although it has been thought that glucose-stimulated AA release occurs via the hydrolysis of membrane phospholipids by PLA_2 enzymes (41-43), another possibility is the hydrolysis of DAG by DAG lipase within the DAG/free fatty acid cycling pathway. PLA_2s liberate free fatty acids from the *sn*-2 position of membrane phospholipids. Cytosolic PLA_2s are divided into two groups: $cPLA_2$ (cytosolic Ca^{2+}-dependent, group IV) and $iPLA_2$ (cytosolic Ca^{2+}-independent, group VI). $cPLA_2$ requires micromolar Ca^{2+} for membrane translocation but not for catalysis and possesses a selectivity towards phospholipids containing the AA moiety (44; 45). $iPLA_2$ exhibits absence of substrate specificity for AA-containing phospholipids and no Ca^{2+} requirement for activity (44; 45). ATP-stimulated $iPLA_2$ isoenzymes have been implicated in glucose-stimulated AA release (42), since glucose activates AA release in the absence of Ca^{2+} (46); also, the pharmacological $iPLA_2$ inhibitor, bromoenol lactone suicide substrate, inhibits AA release and insulin secretion *in vitro* (42; 43). In diabetic GK islets, cholinergic stimulation induces an enhancement of insulin release which is largely mediated through mechanisms involving hydrolysis of DAG to AA (47). It has been shown that AA promotes the redistribution of cytosolic PKC to the membrane in a time- and dose-dependent fashion. This study establish a link between AA generation and PKC activation, and supports the notion that cytosolic PKC may be a downstream target in AA-induced insulin release (48; 49). There are also data showing that stimulation of insulin secretion from islets in response to AA does not require its metabolism through cyclooxygenase-2 and 5-/12-lipooxygenase pathways (50) whilst metabolism of AA to epoxyeicosatrienoic acids through cytochrome P-450 pathway is involved in the stimulation of insulin secretion (51).

1.2. Imidazoline compounds

A number of compounds with an imidazoline structure are effective potentiators of insulin secretion in pancreatic ß-cells (52-57). Phentolamine, an α_2-adrenergic blocking agent with an imidazoline moiety, stimulated basal and glucose-induced insulin release (58). These data were initially interpreted as an indication of the important role of α_2-adrenergic receptors in the regulation of insulin release, even under non-stress conditions. Subsequently, it was demonstrated that the more selective α_2-adrenergic blocking agent idazoxan does not enhance basal or glucose-

stimulated insulin release. Therefore, it was proposed that the stimulatory effect of phentolamine on insulin release could not be accounted for by its action on α_2-adrenoceptors, but to the effect of the compound on other sites (54; 55). Similarly, others have shown that imidazoline substances increased insulin release after irreversible blockade or downregulation of α_2-adrenoceptors, and they proposed that the stimulatory effect of the compounds on insulin release was probably related to their interaction with imidazoline receptors (56; 57; 59).

The imidazoline receptors constitute a class of non-adrenergic binding sites, which possess a high affinity for ligands bearing an imidazoline moiety. Based on the ligand selectivity, there are two identified imidazoline receptor types: I_1-imidazoline receptor which mediates the sympatho-inhibitory actions to lower blood pressure, I_2-receptor which is an important allosteric binding site of the mitochondrial monoamine oxidases A and B.

Previously the insulinotropic effect of imidazoline compound was attributed to the blockade of K_{ATP} channels (60), particularly the Kir6.2, a pore-forming subunit of this channel (61-63). A decade ago the K_{ATP} channel-independent pathway by which imidazoline compounds stimulate insulin secretion was defined in our group (64). It was demonstrated that the imidazoline compound RX871024 promotes insulin release by at least two modes of action (64-66). One mode includes the inhibition of K_{ATP} channels, membrane depolarization and opening of voltage-dependent L-type Ca^{2+} channels. The other, a more distal effect of the imidazoline, affected the exocytotic machinery and was not related to changes in $[Ca^{2+}]_i$ (64-66). Previously, it was shown that the K_{ATP} channel-independent insulinotropic action of RX871024 is dependent on the activity of PKA and PKC (63; 66). This K_{ATP}-independent effect of imidazolines on insulin secretion differs from the K_{ATP} channel-independent sulfonylurea effect, as the latter is not sensitive to PKA inhibition but is PKC-dependent (67). The K_{ATP} channel-independent effect of imidazolines was attributed to their interaction with a putative I_3-imidazoline receptor. A further elaboration of the K_{ATP} channel-independent pathway of imidazoline signaling in pancreatic β-cells led to the suggestion that the I_3-imidazoline receptor is Rhes, a monomeric G-protein. It was reported that Rhes expression was controlled in an efaroxan-sensitive manner and that the Rhes protein is responsible for the direct stimulation of insulin exocytosis by efaroxan (68).

It has also been suggested that imidazolines stimulate insulin secretion through interactions with phencyclidine-binding sites, mechanically blocking the pore of the K_{ATP} channel (69). However, this hypothesis could not be confirmed, since other results showed that phencyclidine and its analogues do not induce insulin secretion (70).

Recently, in our group a pure glucose-dependent insulinotropic imidazoline compound, BL11282 has been developed (Fig. 2) (5; 51). BL11282 does not stimulate insulin secretion at basal glucose concentration whereas it potentiates insulin secretion at elevated glucose level (5; 51). This remarkable effect is explained by the K_{ATP} channel-independent stimulation of insulin release by BL11282 (5; 51), and is dependent on the activities of PKA and PKC (5; 51). It was shown that intravenous administration of BL11282 to anesthetized rats did not change blood glucose and insulin levels under basal conditions, but increased blood insulin levels and glucose removal from the blood after glucose infusion (5).

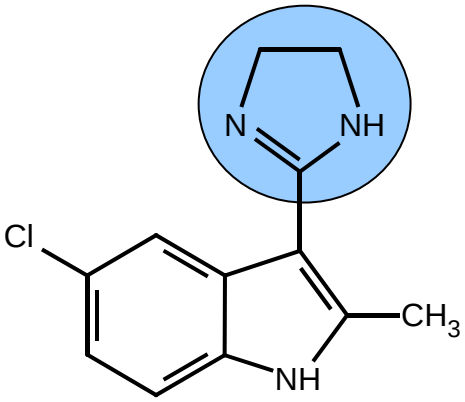

Fig. 2. Chemical structure of imidazoline compound BL11282 (imidazoline moiety is highlighted).

In addition, increases in insulin sensitivity due to treatment with imidazoline compounds was also demonstrated in clinical trials (71; 72).

However, to date, both the binding sites and the molecular mechanism underlying the K_{ATP} channel-independent, direct regulation of insulin exocytosis by imidazolines have not been completely established.

AIMS

Overall objective

The overall objective of this study was to investigate the molecular mechanisms of the pure glucose-dependent insulinotropic activity of the imidazoline compound BL11282.

Specific aims of the present work

1. To create the conditions of long-term incubation of pancreatic islets with BL11282 which influence the insulin secretory response to a subsequent challenge with the same compound.

2. To test whether previously described targets for imidazolines are involved in the insulinotropic activity of the novel imidazoline compound BL11282:
   - K_{ATP} channels;
   - α_2-adrenergic receptors and I_1-imidazoline receptors;
   - monomeric G-protein, Rhes.

3. To compare BL11282-dependent signal transduction pathways leading to stimulation of insulin secretion with those pathways that are involved in the stimulation of insulin secretion by the pure glucose-dependent insulinotropic peptide GLP-1.

4. To investigate the role of arachidonic acid signaling in the mechanism of the K_{ATP} channel-independent stimulation of insulin secretion by imidazoline compound BL11282.

2 EXPERIMENTAL DESIGN, MATERIALS AND METHODS

For studies of the molecular mechanisms of the insulinotropic activity of the novel imidazoline compound BL11282 we have used different experimental approaches listed in Table 1.

Table 1. Experimental design

Study	Experimental approach	Object	Method	Measurement
I	Desensitization	Rat and *ob/ob* mouse islets	Batch-incubation	Insulin secretion
II	SUR1-subunit knock-down Pharmacological inhibitors Desensitization Plasmid transfection	Mouse islets Rat islets Rat islets MIN6 cells	Batch-incubation Batch-incubation RT-PCR Batch-incubation	Insulin secretion Insulin secretion mRNA expression Insulin secretion
III	Desensitization Plasmid transfection	Rat islets MIN6 cells	Batch-incubation Batch-incubation	Insulin secretion Insulin secretion
IV	Pharmacological inhibitors	Rat islets Rat islets Rat islets	RT-PCR Batch-incubation	mRNA expression Insulin secretion Arachidonic acid release

3.1. Reagents

BL11282 (5-chloro-3-(4,5-dihydro-1H-imidazol-2-yl)-2-methylindole hydrochloride) was obtained from Eli Lilly (Indianapolis, IN, USA). Efaroxan (2-[2-(2-ethyl-2,3-dihydrobenzofuranyl)]-2-imidazoline hydrochloride), diazoxide (7-chloro-3-methyl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide), BEL (bromoenol lactone; 2H-pyran-2-one, 6-(bromoethylene)tetrahydro-3-(1-naphthalenyl)-, (E)-), MB-1-ABT (methylbenzyl-1-aminobenzotriazole), AGN192403 (2-endo-amino-3-*exo*-isopropyl-bicyclo[2.2.1] heptane hydrochloride), yohimbine (17α-hydroxy-yohimban-16α-carboxylic acid methyl ester) and β-mercaptoethanol were purchased from Sigma (St. Louis, MO, USA). D609 (tricyclodecan-9-yl xanthate), AACOCF$_3$ (arachidonyl trifluoromethyl ketone) and BBPA (N-{(2S,4R)-4-(biphenyl-2-ylmethyl-isobutyl-amino)-1-[2-(2,4-difluorobenzo-yl)-benzoyl]-pyrrolidin-2-ylmeth-yl}-3-[4-(2,4-dioxi-thiazolidin-5-ylidenemethyl)-phenyl]acrylamide hydrochloride) were purchased from Calbiochem (San Diego, CA, USA). GLP-1 was from Polypeptide Laboratories

GmbH (Wolfenbüttel, Germany). RPMI-1640 medium, fetal calf serum, penicillin, streptomycin sulfate, trypsin and glutamine were obtained from Gibco (Paisley, UK). Rat insulin was from Novo Nordisk (Denmark). All other reagents were of analytical grade.

3.2. Isolation of pancreatic islets

The Ethics Committee on Animal Research in Northern Stockholm approved all experimental procedures. 2-3-months old Wistar rats were obtained from B&K Universal (Sollentuna, Sweden). 2-3-months old GK rats and 10-12 months old non-diabetic *ob/ob* mice were from a local colony at Karolinska Institutet. The mice lacking the SUR1 receptor ($SUR1^{(-/-)}$ mice; 2-4 months of age) were obtained from Prof. M. Magnuson (Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, Tennessee, USA) (31). Islets from mice and rats were isolated by collagenase digestion (73). Isolated rat pancreatic islets were incubated with or without imidazoline compounds at 37 °C for 18-22 h in a humidified atmosphere of 5% CO_2, in RPMI-1640 medium (5.5 or 11 mM glucose) supplemented with 10% FCS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin sulphate.

3.3. β-cell line

The β-cell line MIN6 (passages 32–38) was maintained in DMEM containing 25 mM glucose, supplemented with 10% fetal calf serum, 50 U/ml penicillin, 0.05 mg/ml streptomycin sulfate, and 50 μM β-mercaptoethanol, in a humidified atmosphere of 5% CO_2 at 37 °C. MIN6 monolayers were trypsinized (0.1% trypsin, 0.02% EDTA) at 80–90% confluency and were plated in 24-well plates 24 h before transfection.

3.4. Plasmids

Plasmid pHG327.Rhes, which contained the cDNA for Rhes, was kindly provided by Prof. J. Gregor Sutcliffe (The Scripps Research Institute, La Jolla, CA, USA). The cleavage product of pHG327.Rhes digestion with *Nar*I was incubated with Klenow polymerase and then digested with *Apa*I. The resulting Rhes cDNA

fragment was inserted into the *Eco*RV- and *Apa*I-digested construct pRcCMVi.EGFP (74), thereby replacing the EGFP cDNA with Rhes cDNA to generate pRcCMVi.Rhes. To obtain the Rhes-antisense expression construct, an 800 bp Rhes-fragment was obtained by digesting pRcCMVi.Rhes with *Sma*I and *Apa*I. This DNA fragment was subcloned into *Hpa*I- and *Apa*I-digested pB.rIns1.DsRed (75), thereby replacing the DsRed cDNA and generating pB.rIns1.Rhes-antisense. This permitted the Rhes cDNA fragment to be placed in the antisense orientation under control of the rat insulin-1 promoter (−410/+1). All vector constructions were verified by DNA sequence analysis. Plasmids pSRα-cAMP-GEFII (G114E, G422D), and mutant pCMV-HA-Rim2ΔA (193-830) were kindly provided by Prof. Susumi Seino (Department of Cellular and Molecular Medicine, Chiba University, Japan).

3.5. Transfection

MIN6 cells were transfected with pRcCMVi.EGFP, pRcCMVi.Rhes, pB.rIns1.EGFP, pB.rIns1.Rhes-antisense, mutant pSRα-cAMP-GEFII (G114E, G422D), and mutant pCMV-HA-Rim2ΔA (193-830) plasmids in the presence of LipofectAMINE 2000 (Invitrogen–Life Technologies, CA, USA), according to the manufacturer's instructions. Transfection efficiency was estimated by microscopic evaluation of EGFP fluorescence with an inverted microscope (Zeiss Axiovert 133TV; Carl Zeiss MicroImaging). Excitation light was obtained from a SPEX fluorolog-2 MM1T11I spectrofluorometer (Spex Industries). The following settings were used for EGFP detection: excitation at 485 nm, a 505-nm dichroic mirror, and a 505–535-nm band-pass emission filter. Measurements of insulin secretion from MIN6 cells were performed 72 h after transfection, when transfection efficiency was maximal.

3.6. Measurements of insulin secretion

Insulin secretion from islets was measured in KRBB containing (in mM): 115 NaCl, 4.7 KCl, 2.6 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 20 $NaHCO_3$, 16 HEPES and 2 mg/ml BSA; pH 7.4. Islets were preincubated in KRBB with 3.3 mM glucose at 37 °C for 1 h. Islets were preincubated in KRBB with 3.3 mM glucose at 37°C for 30 min, then for the following 30 min the respective test substances: yohimbine,

AGN192403, D609, AACOCF$_3$, BBPA, BEL and MB-1-ABT were added to the preincubation medium. Groups of three islets were incubated at 37 °C for 1 h in 300 µl of the same buffer containing 3.3 or 16.7 mM glucose, or 16.7 mM glucose and the respective test compound. Insulin secretion from MIN6 cell was measured in EBSS containing (in mM): 115 NaCl, 5.3 KCl, 1.8 $CaCl_2$, 1.0 NaH_2PO_4, 0.8 $MgSO_4$, 26 $NaHCO_3$, and 1 mg/ml BSA; pH 7.4. MIN6 cell were preincubated in EBSS with 1 mM glucose at 37 °C for 1 h. Cells were then incubated at 37 °C for 1 h in 500 µl of the same buffer containing 1 or 25 mM glucose, or 25 mM glucose plus 50 µM BL11282. Supernatants from the incubations were chilled on ice and aliquoted prior to measurement of insulin by radioimmunoassay, employing rat insulin as standard.

3.7. Measurements of arachidonic acid release

Generation of arachidonic acid was quantified by efflux of [^{3}H]arachidonic acid from [^{3}H]arachidonic acid-prelabeled islets, as previously described (47; 76). Isolated islets were incubated overnight in batches of 25 at 37°C in a humidified atmosphere of 5% CO_2 in RPMI-1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin sulfate and 0.2 µCi [^{3}H]arachidonic acid (specific activity, 180-240 Ci/mmol; PerkinElmer Life and Analytical Sciences). After incubation, the islets were washed three times with KRBB containing 1 mg/ml BSA with 3.3 mM glucose and devoid of [^{3}H]arachidonic acid and then preincubated for 30 min at 37 °C with or without 25 µM BEL in 1 ml of the same buffer in a humidified atmosphere of 5% CO_2. Subsequently, the islets were incubated under the same conditions at 16.7 mM glucose with or without BL11282 and BEL for 30 min. Following the incubation period, the incubation buffer was removed and radioactivity was determined by liquid scintillation counting using scintillation cocktail ULTIMA GOLD (PerkinElmer Life and Analytical Sciences, USA). The radioactivity of the islets was also determined. [^{3}H]arachidonic acid release was estimated as the radioactivity in the removed buffer divided by the total islet radioactivity.

3.8. RNA extraction

Islets were collected under a stereo-microscope and employed immediately for RNA extraction, using RNeasy RNA purification kit (Qiagen, Germany),

according to the manufacturer's instructions. RNA was treated with DNase I (Qiagen, Germany) for 15 min at room temperature. RNA concentration was measured by 260 nm absorbance using a conversion factor of 40. Quality and integrity of RNA (1 μg per line) was detected by agarose gel electrophoresis in sodium phosphate buffer. RNA samples were stored at -80°C.

3.9. Semi-quantitative RT-PCR

Reverse transcription was carried out using SuperScript II First-Strand Synthesis System (Invitrogen–Life Technologies, CA, USA) according to the manufacturer's instructions in reactions containing 1.5 μg total RNA, 0.5 mM dNTPs, 150 ng random hexamer primers, 5 mM $MgCl_2$, 0.01 M dithiothreitol, and 40 U RNaseOut Recombinant Inhibitor (Invitrogen–Life Technologies, CA, USA) in a final volume of 20 μl as described (77). The template was denatured by heating (65°C for 5 min) and annealing at 25°C for 12 min. The reverse transcription reaction was run at 42°C for 50 min followed by enzyme inactivation at 70°C for 15 min. Aliquots of each reverse transcription mix removed prior to the addition of reverse transcriptase served as negative controls. Semi-quantitative PCR was performed independently of cDNA samples generated from four experiments. PCR conditions were chosen such that the amplification of analyzed gene fragments were within the linear range. This was verified by testing various numbers of amplification cycles (paper II and III). PCR was carried out in 10 μl reactions containing 4 μM dNTPs, 2.5 mM $MgCl_2$, 5 pmol forward and reverse specific primers, 0.4 U *Taq* DNA polymerase (Roche, Switzerland), and quantities of cDNA corresponding to 10 or 50 ng total RNA. The sequences of the primers used are shown in Table 2. The specific primers for rPL30 and β-actin in rat islets were used as internal controls. PCR products were analyzed by electrophoresis on 1.5% agarose gels; bands were visualized with ethidium bromide staining and documented with a digital camera (EDAS 290, Kodak) and software (1D, Kodak). All PCRs included reverse transcription-negative controls, and these reactions consistently yielded no amplification product. PCR products generated from pancreatic islet cDNA with corresponding primers (see Table 2) were gel-purified (NucleoTrap, Clontech), cloned with a TOPO-TA Cloning kit (Invitrogen-Life Technologies, CA, USA) and

sequenced with a ABI Prism BigDye® Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, CA, USA), as recommended by the manufacturer.

Table 2. Primer sequences for RT-PCR

Target gene (Accession number)	**Sense primer sequence**	**Antisense primer sequence**
rPL30 (K02932)	GGAAAGTACGTGCTGGGG TA	CACCTGGGTCAATGATAG CC
β-Actin (NM_031144)	TGTGCCCATCTACGAGGGGTA TGC	GGTACATGGTGGTGCCGCCAG ACA
Rhes (NM_133568)	GCAAGAGCTCCATTGTCTCC	CGTGTTCTTCTTGGCTGACA
$iPLA_2\beta$ (RGD: 628867)	CAGAGAATGAGGAGGGCTGT	GGATCCTTGCTGTGGATCTG

Specific clones were identified by restriction digestion of plasmid preparations (Qiaprep, Qiagen) with *EcoR*I. The sequence was determined by using an ABI 373 automated DNA sequencer (Applied Biosystems, CA, USA). Assembly and alignment of the sequencing data were performed with GeneWorks 5.2 (Oxford Molecular, UK). Data sets were analyzed using the NCBI BLAST. Comparison between sequences deposited at the public database GenBank and sequences derived as described above was performed using the software BLASTN.

3.10. Statistical analysis

Data was analyzed using Sigma Plot for Windows (version 7.101, SPSS, Inc.), Statistica (version 5.0, StatSoft, Inc). All results are expressed as means ± SE for the indicated number of experiments. Analysis of mean values was estimated with Student's *t* test or one-way ANOVA followed by the LSD test. Differences between mean values were considered significant if $p < 0.05$.

RESULTS AND DISCUSSION

4.1. Prolonged incubation with BL11282 desensitizes pancreatic islets to further stimulation by this imidazoline compound but leads to an increase in insulin secretion at high glucose concentration (paper I).

The elaboration of the molecular mechanisms underlying the effect of BL11282 on insulin secretion is complicated by the absence of a selective antagonist of this compound. Therefore, creating conditions where BL11282 does not possess insulinotropic activity and comparing them to those conditions where BL11282 stimulates insulin secretion can give a hint to the signal-transduction pathways involved. In this study we have used an approach involving desensitization of ß-cells to the insulinotropic activity of BL11282 after prolonged incubation with the compound. We have evaluated how the conditions of long-term exposure of pancreatic islets to BL11282 influenced the insulin secretory response to a subsequent challenge with the same compound. Then we have compared the BL11282-dependent signal-transduction pathways with the signal-transduction pathways involved in the stimulation of insulin secretion by the pure glucose-dependent insulinotropic peptide GLP-1.

We have identified conditions of incubation with BL11282 which lead to desensitization of the subsequent response to this imidazoline. Pretreatment with 50 µM BL11282 for 18-21 h desensitized Wistar rat and *ob/ob* mouse islets to BL11282. These desensitization conditions have been used for studies of the mechanisms underlying the insulinotropic activity of BL11282 (study III).

We have compared the desensitization effects of the two imidazolines BL11282 and efaroxan on subsequent stimulation of insulin release by high glucose concentration in pancreatic islets. Efaroxan is an imidazoline compound of the first generation (51) which stimulates insulin secretion from pancreatic islets by two modes of action, i.e. by the K_{ATP} channel-dependent mechanism involving binding to the Kir6.2 subunit of the K_{ATP} channel (60) and, as it was later shown, the K_{ATP} channel-independent mechanism by affecting distal components of the exocytotic machinery (78). At 16.7 mM glucose the K_{ATP} channels are almost fully blocked by the high ATP/ADP ratio (11) and glucose-induced insulin secretion is almost maximal. Therefore, the observed effects of efaroxan on insulin secretion at high

glucose concentration can mainly be attributed to the K_{ATP} channel-independent pathway.

Imidazoline compounds of the second generation, e.g. BL11282, stimulates insulin release in pancreatic islets only through the K_{ATP} channel-independent mechanism (5; 51). To elaborate possible interactions between BL11282 and efaroxan signaling pathways in their K_{ATP} channel-independent mode of action we have pre-incubated pancreatic islets with efaroxan and then investigated the islet response to both imidazoline compounds. Results of these studies demonstrate that after exposure of islets to efaroxan they remained significantly responsive to BL11282 but not to efaroxan. The data obtained suggest that BL11282 and efaroxan desensitize pancreatic islets by alternative pathways and stimulate insulin secretion by different K_{ATP} channel-independent mechanisms.

An interesting observation is that after prolonged culture with BL11282, pancreatic islets from Wistar rats and *ob/ob* mice were able to release higher amounts of insulin in response to a subsequent glucose challenge compared to islets cultured without BL11282. This increase in glucose-induced insulin secretion favors the conclusion that BL11282-induced desensitization does not lead to depletion of releasable insulin stores. BL11282-desensitized islets are even able to further release significant amounts of insulin after subsequent stimulation with other secretagogues, as was demonstrated with GLP-1.

Interestingly, prolonged exposure of islets to efaroxan (79), or sulphonylurea (80-82) does not sensitize islets to subsequent glucose stimulation. In our experiments it was shown, that in contrast to BL11282, pre-incubation of islets with efaroxan had even a tendency to decrease insulin response to a subsequent glucose challenge. This effect of efaroxan is in full agreement with a previous study (79). The authors showed a decrease in the amount of insulin granules and an increased level of degranulated β-cells after culture of islets with efaroxan or with sulphonylurea (79).

The increase in glucose-induced insulin secretion following the prolonged incubation with BL11282 can be explained by changes in the expression pattern of a number of proteins, which are observed under the very same conditions (83). Indeed as it has been shown in our previous studies (83) prolonged incubation of rat pancreatic islets with BL11282, leading to desensitization of the insulin response to the compound, is accompanied by an increase in proteins involved in protein folding (e.g. Hsp60, protein disulfide isomerase and calreticulin), metabolism (e.g. pyruvate

kinase, α-enolase, transketolase, isocytrate dehydrogenase and 3-ketoacyl-CoA thiolase), and exocytosis (e.g. calcyclin and Annexin I) (83).

4.2. The role of previously described targets for imidazolines in BL11282-dependent stimulation of insulin secretion (papers II, III).

In this study we aimed to examine whether previously described targets for imidazolines (K_{ATP} channels, α_2-adreno- and I_1-receptors and monomeric G-protein Rhes) are involved in stimulation of insulin secretion by BL11282. In order to evaluate the role of these targets we have used different experimental approaches, i.e. desensitization, plasmid transfection and pharmacological inhibitors.

It has previously been shown that insulinotropic properties of imidazoline compound BL11282 were not related to activation of K_{ATP} channels. For additional verification of this fact we have examined a mouse model with a deletion of the regulatory subunit, SUR1, of K_{ATP} channels (31) (paper II). Removing the SUR1 subunits blocks the assembly of Kir6.2 subunits into a functionally active channel and transport from the ER (84; 85). Hence, insulin release in $SUR1^{(-/-)}$ islets stimulated by BL11282 can not be attributed to the interaction of BL11282 with K_{ATP} channels. Indeed, we have observed that BL11282 potentiated insulin secretion at a stimulatory glucose level in islets from $SUR1^{(-/-)}$ knockout mice. These findings unambiguously confirm the K_{ATP} channel-independent, glucose-dependent direct effect of BL11282 on insulin exocytosis.

Antagonism of α_2-adrenoreceptors was believed to be the mechanism of action of the imidazoline compound phentolamine (52; 53). To evaluate whether BL11282 possesses the α_2-adrenergetic activity we used the α_2-adrenergic antagonist yohimbine. (paper II). Our experiments clearly showed that yohimbine itself or in combination with BL11282 did not alter insulin secretion in pancreatic islets. Therefore, it can be concluded that α_2-adrenoreceptors are not involved in BL11282-mediated insulin secretion.

It has been suggested that some imidazolines can interact with imidazoline I_1-receptors, activating PC-PLC (86; 87). This suggestion was based on the finding that a selective I_1-receptor agonist, moxonidine, increased the concentration of DAG and phosphocholine in neurons and PC12 cells (87). An inhibitor of PC-PLC, D609, blocked moxonidine effect on DAG concentration (87). To examine whether

imidazoline BL11282 is involved in a signaling pathway coupled to I_1-receptor/PC-PLC we have used a selective I_1-receptor antagonist AGN192403 (88) and PC-PLC inhibitor D609 (89) (paper II). The data obtained show that both blockage of I_1-receptor with AGN192403 and PC-PLC with D-609 do not affect insulin secretion induced by BL11282. These observations do not support the involvement of imidazoline I_1-receptors and PC-PLC in the stimulatory effect of BL11282 on insulin secretion.

It has been suggested that a monomeric G-protein, Ras homologue expressed in striatum (Rhes) (90), is an imidazoline-regulated protein (68) that is involved in the K_{ATP} channel-independent stimulation of insulin secretion by the imidazoline derivative efaroxan (paper III). This suggestion was based on observations regarding changes in Rhes gene mRNA expression in rat islets and pancreatic β-cells after prolonged culture with efaroxan. In our study, to test whether the Rhes protein is involved in the regulation of the insulin secretion process by imidazoline compound BL11282, we have evaluated the effect of this imidazoline on Rhes mRNA expression in isolated rat islets maintained under conditions identical to those used by Chan et al. (68). In addition, we have evaluated the influence of BL11282 on insulin secretion in MIN6 cells transfected with Rhes and Rhes-antisense plasmids. As a reference compound in all experiments we have used efaroxan.

Prolonged culture (18 h) of pancreatic islets with 100 μM efaroxan or 50 μM BL11282 under, the same conditions as those used by Chan et al. (68), caused desensitization of the islet response to the imidazoline compound, which is in agreement with the previous report (68). In the study by Chan et al. (68), it was also shown that desensitization of pancreatic islets to efaroxan was accompanied by a significant decrease in Rhes mRNA expression. To verify this effect, we have carried out semi-quantitative RT-PCR evaluation of Rhes mRNA levels in islets desensitized either to efaroxan or BL11282, in comparison to control islets. mRNA of Rhes gene is expressed in rat pancreatic islets but no significant changes in Rhes gene expression could be detected in either efaroxan- or BL11282-treated islets, compared to control.

For an additional verification of the putative role for Rhes in the insulinotropic activity of imidazolines, we have used an alternative approach involving either over-expression of Rhes or its down-regulation by a Rhes-antisense construct. The transfection of MIN6 cells with plasmids containing Rhes or Rhes-antisense does not affect either efaroxan- or BL11282-induced insulin secretion. In

addition, up- or down-regulation of Rhes has no effect on glucose-stimulated insulin release. Hence, the data obtained do not confirm the suggestion that Rhes is an imidazoline-regulated protein and are not consistent with the proposal that the Rhes protein is responsible for the direct stimulation of insulin exocytosis by imidazoline compounds efaroxan and BL11282.

4.3. Comparison of BL11282-dependent signal-transduction with those pathways that are involved in the stimulation of insulin secretion by GLP-1 (paper I).

Imidazoline compound BL11282 has been developed as an oral compound possessing pure glucose-dependent insulinotropic activity, similar to the peptide GLP-1. The cAMP system plays a key role in the mechanism of a pure glucose-dependent insulinotropic activity of GLP-1. GLP-1 is a peptide which interacts with G-protein coupled receptors and activates the G_s-adenylate cyclase-cAMP signaling pathway (33; 34). This leads to an increase in cAMP production and thereby stimulation of PKA and the cAMP-GEF signaling pathway involved in insulin exocytosis (91; 92). Therefore, it was important to evaluate whether and to what extent signal-transduction pathways of BL11282 coincide with the GLP-1 pathway. Desensitization of islets to BL11282 does not abolish the ability of GLP-1 to stimulate glucose-induced insulin release under conditions where the peptide is added both in the absence and in the presence of BL11282 (paper I, Fig. 3A).

However, islet desensitization to the imidazoline leads to a significant decrease in the fold of potentiation of glucose-induced insulin release by GLP-1. In BL11282-treated islets this fold stimulation is twice less than in control islets (paper I, Table 1). This may suggest that BL11282 affects signal-transduction pathways involved in the effects of GLP-1 on insulin secretion, i.e. cAMP/PKA/cAMP-GEFII·Rim2 pathways, and prolonged incubation with the imidazoline leads to down-regulation of these pathways. We have previously shown that BL11282-stimulated insulin secretion is dependent on PKA activity (5).

The data on the effect of expression of dominant-negative cAMP-GEFII and Rim2 (paper I, Fig. 3B) point to the importance of the cAMP-GEFII·Rim2 pathway in BL11282-stimulated insulin secretion. Indeed, expression of dominant negative cAMP-GEFII (G114E, G422D) and Rim2ΔA mutant protein in MIN6 cells led to a significant reduction in insulin secretion stimulated by the imidazoline.

4.4. Arachidonic acid signaling and stimulation of insulin secretion by BL11282 (paper II).

BL11282 was demonstrated to stimulate insulin exosytosis in islets at steps distal to the rise in $[Ca^{2+}]_i$ (5; 51). There are a number of suggestions in the literature that arachidonic acid pathways are involved in the regulation of insulin secretion from pancreatic β-cells, which takes place without concomitant increases in $[Ca^{2+}]_i$ (93). These pathways include either intact arachidonic acid or its biologically active metabolites generated by cytochrome P-450, leading to epoxyeicosatrienoic acids (94; 95). In pancreatic islets arachidonic acid is released from phospholipids (mediated by PLA_2s activity) (44; 45). To evaluate the role of these pathways in BL11282-stimulated insulin secretion we have used the inhibitors of these enzymes.

We have evaluated the effects of $cPLA_2$ and $iPLA_2$ inhibitors on BL11282-stimulated insulin release under normal and depolarized conditions in pancreatic islets. We used the inhibitors of $cPLA_2$, $AACOCF_3$ and BBPA. Under normal conditions, the inhibitors $AACOCF_3$ and BBPA did not affect glucose-stimulated insulin secretion, while only partial suppression of BL11282-induced insulin release was observed in the presence of $AACOCF_3$ and BBPA inhibitors. However, under depolarized conditions, when $[Ca^{2+}]_i$ was clamped, BBPA did not show any inhibitory effect on BL11282-stimulated insulin secretion. Hence, this data indicate that $cPLA_2$ activity is not required for the direct, independent of $[Ca^{2+}]_i$ changes, effect of BL11282 on insulin secretion.

To further investigate this direct mechanism of BL11282 on insulin release, we turned our attention to the $[Ca^{2+}]_i$-independent PLA_2 isoform $iPLA_2\beta$, which is predominantly expressed in pancreatic islets and plays an important role in insulin secretion in pancreatic islets and insulinoma cells (96). Our observations indicate a deficiency in $iPLA_2\beta$ isoform expression in diabetic GK rat islets compared to Wistar rat islets, this effect being in agreement with an impaired insulin response in GK rat islets (97). Therefore, these findings support the idea that $iPLA_2\beta$ is an important player in insulin secretion and reduction in $iPLA_2\beta$ expression can be one of the causative factors of impaired insulin secretion under diabetic conditions. Addition of BL11282 fully normalizes glucose-induced insulin release in pancreatic islets from diabetic GK rats (51).

The results with the use of BEL (bromoenol lactone), an inhibitor of $iPLA_2$, also point to the importance of the enzyme in the insulinotropic activity of BL11282. Although BEL partially inhibits insulin release stimulated by high glucose concentration under depolarized conditions when $[Ca^{2+}]_i$ is clamped (paper II, Fig. 4B), a significant stimulation of insulin release by glucose is still present. However, the presence of BEL completely blocked BL11282-induced potentiation of glucose-induced insulin release. In the presence of BEL, the levels of stimulation of insulin release by glucose either in the absence or presence of BL11282 are the same (paper II, Fig. 4B). Hence arachidonic acid generation through the $iPLA_2$ pathway is necessary for the potentiation of glucose-stimulated insulin secretion by the imidazoline. Indeed, BL11282 stimulated arachidonic acid release from the islets in the presence of high glucose concentration and this effect was fully blocked by BEL (paper II, Fig. 5). Thus, BL11282 effects on insulin secretion, occurring independently from concomitant changes in $[Ca^{2+}]_i$, can be attributed to mechanisms involving $iPLA_2$ activity.

Cytochrome P-450 generated epoxyeicosatrienoic acids have been shown to play a role in glucose-induced insulin secretion (98). In our previous work we have demonstrated that there is a suppressive effect of the cytochrome P-450 inhibitor MB-1-ABT (99) on insulin secretion induced by glucose and imidazoline compound RX871024 (51). We have evaluated the effect of the cytochrome P-450 inhibitor MB-1-ABT (99) on insulin secretion induced by glucose and BL11282. Incubation with MB-1-ABT partially inhibited glucose-induced insulin secretion. However, the inhibitor fully suppressed imidazoline-induced potentiation of glucose-stimulated insulin secretion. In the presence of MB-1-ABT the level of stimulation of insulin release by high glucose concentration is the same both in the absence or presence of BL11282 (paper II, Fig. 4C). These observations suggest that arachidonic acid metabolism by cytochrome P-450, leading to epoxyeicosatrienoic acids, is important in potentiation of glucose-induced insulin release by the imidazoline compound BL11282.

In conclusion, the results of this study suggest that potentiation of glucose-induced insulin release by BL11282, independent of concomitant changes in $[Ca^{2+}]_i$, involves release of arachidonic acid by $iPLA_2$ and its metabolism to epoxyeicosatrienoic acids through the cytochrome P-450 pathway.

3 CONCLUSIONS

The molecular mechanisms underlying the stimulatory effect of a novel pure glucose-dependent imidazoline derivative BL11282 on insulin secretion were defined in this study. The following conclusions can be made:

- Using $SUR1^{(-/-)}$ mice, we unambiguously confirmed the previous notion that the insulinotropic activity of BL11282 is unrelated to its interaction with ATP-dependent K^+ channels.

- BL11282 acts by mechanisms distinct from involving α_2-adrenoreceptors, imidazoline I_1-receptors and imidazoline I_1-receptor coupled PC-PLC activation.

- Our studies do not confirm the suggestion that Rhes is an imidazoline-regulated protein and are not consistent with the proposal that the Rhes protein is responsible for the direct stimulation of insulin exocytosis by imidazoline compounds efaroxan and BL11282.

- The results of our investigation point to the importance of the cAMP-GEFII·Rim2 pathway in the effects of the pure insulinotropic imidazoline compound BL11282.

- Potentiation of glucose-induced insulin release by BL11282, independent of concomitant changes in $[Ca^{2+}]_i$, involves release of arachidonic acid by $iPLA_2$ and its metabolism to epoxyeicosatrienoic acids through the cytochrome P-450 pathway.

6 SUMMARY

In this study we investigated signal-transduction pathways involved in the mechanisms of the pure glucose-dependent insulinotropic activity of the novel imidazoline compound BL11282. In pancreatic β-cells imidazoline compound BL11282 affects a number of targets involved in the regulation of insulin secretion (Fig. 3).

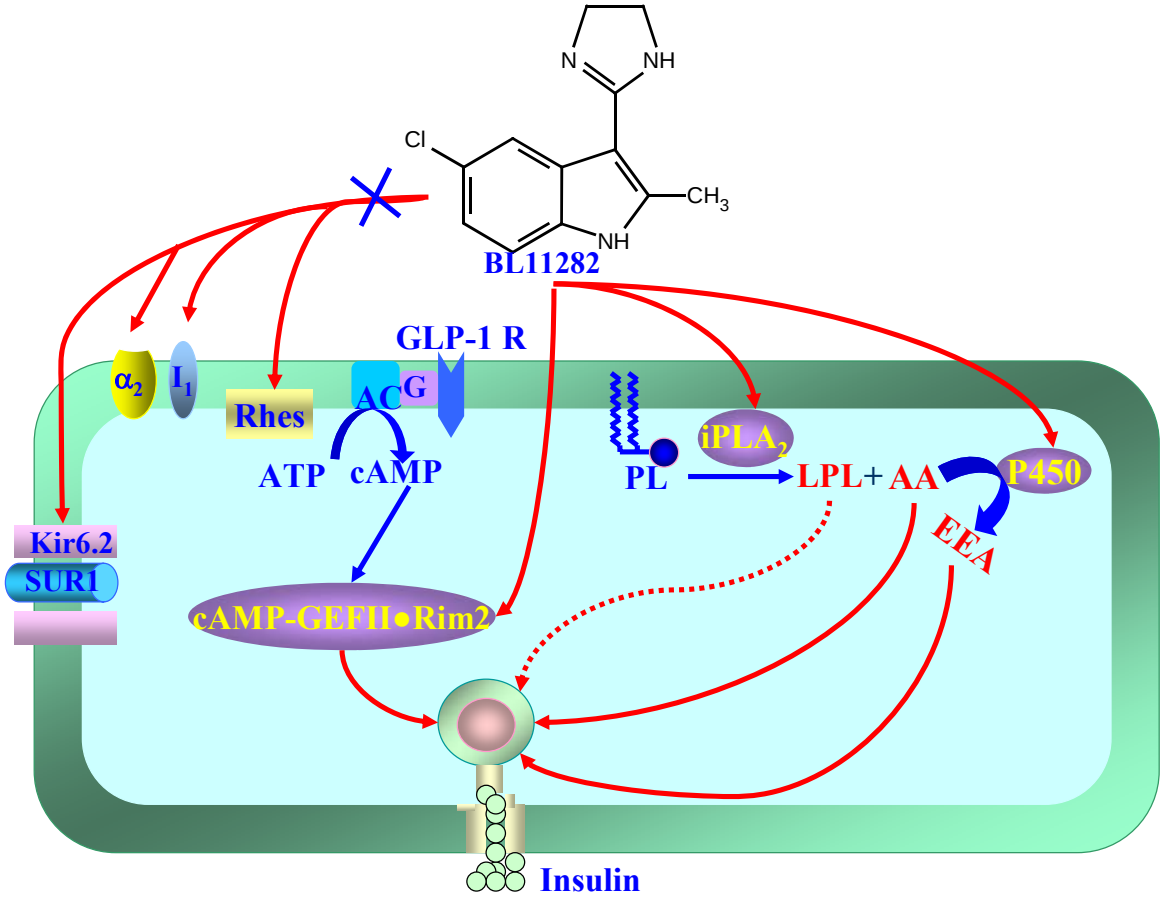

Fig. 3. Signal-transduction pathways involved in the mechanisms of the insulinotropic activity of BL11282 in pancreatic β-cells.

Kir6.2 and SUR1 subunits of ATP-dependent K^+ channel; α_2, α_2-adrenoreceptor; I_1, I_1-imidazoline receptor; Rhes, monomeric G-protein, Ras homologue expressed in striatum; AC, adenylate cyclase; GLP-1 R, GLP-1-receptor; GEFII, cAMP-regulated guanine nucleotide exchange factor II; Rim2, Rab3 interacting molecule; $iPLA_2$, Ca^{2+}-independent phospholipase A_2; PL, phospholipids; LPL, lysophospholipids; AA, arachidonic acid; EEA, epoxyeicosatrienoic acids; P450, cytochrome P-450

The insulinotropic effect of BL11282 is unrelated to its interaction with the previously described targets for imidazolines, i.e.: ATP-dependent K^+ channels, α_2-adrenoreceptors, I_1-imidazoline receptors and monomeric G-protein Rhes. cAMP-GEFII•Rim2 pathway is important in BL11282-stimulated insulin secretion. The insulinotropic effect of BL11282, independent on concomitant changes in $[Ca^{2+}]_i$, involves release of arachidonic acid by $iPLA_2$ and its metabolism to epoxyeicosatrienoic acids through the cytochrome P-450 pathway.

In addition, BL11282 improves β-cells sensitivity to glucose. Results of the present study suggest that imidazoline compounds of the second generation, like BL11282, may be considered as the potential drugs for type 2 diabetes treatment.

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7 REFERENCES

1. Ferrannini E: Insulin resistance versus insulin deficiency in non-insulin-dependent diabetes mellitus: problems and prospects. *Endocr Rev* 19:477-490, 1998
2. Ferner RE, Neil HA: Sulphonylureas and hypoglycaemia. *Br Med J (Clin Res Ed)* 296:949-950, 1988
3. Nauck MA, Meier JJ: Glucagon-like peptide 1 and its derivatives in the treatment of diabetes. *Regul Pept* 128:135-148, 2005
4. Gutniak M, Orskov C, Holst JJ, Ahren B, Efendic S: Antidiabetogenic effect of glucagon-like peptide-1 (7-36) amide in normal subjects and patients with diabetes mellitus. *N Engl J Med* 326:1316-1322, 1992
5. Efanov AM, Zaitsev SV, Mest HJ, Raap A, Appelskog IB, Larsson O, Berggren PO, Efendic S: The novel imidazoline compound BL11282 potentiates glucose-induced insulin secretion in pancreatic beta-cells in the absence of modulation of K_{ATP} channel activity. *Diabetes* 50:797-802, 2001
6. Ashcroft FM, Proks P, Smith PA, Ammala C, Bokvist K, Rorsman P: Stimulus-secretion coupling in pancreatic beta cells. *J Cell Biochem* 55 Suppl:54-65, 1994
7. Berggren PO, Larsson O: Ca^{2+} and pancreatic B-cell function. *Biochem Soc Trans* 22:12-18, 1994
8. Gembal M, Gilon P, Henquin JC: Evidence that glucose can control insulin release independently from its action on ATP-sensitive K^+ channels in mouse B cells. *J Clin Invest* 89:1288-1295, 1992
9. Aizawa T, Sato Y, Ishihara F, Taguchi N, Komatsu M, Suzuki N, Hashizume K, Yamada T: ATP-sensitive K^+ channel-independent glucose action in rat pancreatic beta-cell. *Am J Physiol* 266:C622-627, 1994
10. Zaitsev SV, Efendic S, Arkhammar P, Bertorello AM, Berggren PO: Dissociation between changes in cytoplasmic free Ca^{2+} concentration and insulin secretion as evidenced from measurements in mouse single pancreatic islets. *Proc Natl Acad Sci U S A* 92:9712-9716, 1995
11. Ashcroft FM, Harrison DE, Ashcroft SJ: Glucose induces closure of single potassium channels in isolated rat pancreatic beta-cells. *Nature* 312:446-448, 1984
12. Cook DL, Hales CN: Intracellular ATP directly blocks K^+ channels in pancreatic B-cells. *Nature* 311:271-273, 1984
13. Inagaki N, Gonoi T, Clement JPt, Namba N, Inazawa J, Gonzalez G, Aguilar-Bryan L, Seino S, Bryan J: Reconstitution of IK_{ATP}: an inward rectifier subunit plus the sulfonylurea receptor. *Science* 270:1166-1170, 1995
14. Prentki M: New insights into pancreatic beta-cell metabolic signaling in insulin secretion. *Eur J Endocrinol* 134:272-286, 1996
15. Maechler P, Wollheim CB: Mitochondrial glutamate acts as a messenger in glucose-induced insulin exocytosis. *Nature* 402:685-689, 1999
16. MacDonald MJ: Feasibility of a mitochondrial pyruvate malate shuttle in pancreatic islets. Further implication of cytosolic NADPH in insulin secretion. *J Biol Chem* 270:20051-20058, 1995
17. Brun T, Roche E, Assimacopoulos-Jeannet F, Corkey BE, Kim KH, Prentki M: Evidence for an anaplerotic/malonyl-CoA pathway in pancreatic beta-cell nutrient signaling. *Diabetes* 45:190-198, 1996
18. Deeney JT, Gromada J, Hoy M, Olsen HL, Rhodes CJ, Prentki M, Berggren PO, Corkey BE: Acute stimulation with long chain acyl-CoA enhances exocytosis in insulin-secreting cells (HIT T-15 and NMRI beta-cells). *J Biol Chem* 275:9363-9368, 2000
19. Metz SA, Rabaglia ME, Stock JB, Kowluru A: Modulation of insulin secretion from normal rat islets by inhibitors of the post-translational modifications of GTP-binding proteins. *Biochem J* 295 (Pt 1):31-40, 1993
20. Kowluru A, Chen HQ, Tannous M: Novel roles for the rho subfamily of GTP-binding proteins in succinate-induced insulin secretion from betaTC3 cells: further evidence in support of the succinate mechanism of insulin release. *Endocr Res* 29:363-376, 2003
21. Yamada S, Komatsu M, Sato Y, Yamauchi K, Aizawa T, Kojima I: Nutrient modulation of palmitoylated 24-kilodalton protein in rat pancreatic islets. *Endocrinology* 144:5232-5241, 2003

22. Chapman ER, Blasi J, An S, Brose N, Johnston PA, Sudhof TC, Jahn R: Fatty acylation of synaptotagmin in PC12 cells and synaptosomes. *Biochem Biophys Res Commun* 225:326-332, 1996
23. Gonzalo S, Greentree WK, Linder ME: SNAP-25 is targeted to the plasma membrane through a novel membrane-binding domain. *J Biol Chem* 274:21313-21318, 1999
24. Yajima H, Komatsu M, Yamada S, Straub SG, Kaneko T, Sato Y, Yamauchi K, Hashizume K, Sharp GW, Aizawa T: Cerulenin, an inhibitor of protein acylation, selectively attenuates nutrient stimulation of insulin release: a study in rat pancreatic islets. *Diabetes* 49:712-717, 2000
25. Cheng H, Straub SG, Sharp GW: Protein acylation in the inhibition of insulin secretion by norepinephrine, somatostatin, galanin, and PGE2. *Am J Physiol Endocrinol Metab* 285:E287-294, 2003
26. Straub SG, Sharp GW: Inhibition of insulin secretion by cerulenin might be due to impaired glucose metabolism. *Diabetes Metab Res Rev* 23:146-151, 2007
27. Antinozzi PA, Segall L, Prentki M, McGarry JD, Newgard CB: Molecular or pharmacologic perturbation of the link between glucose and lipid metabolism is without effect on glucose-stimulated insulin secretion. A re-evaluation of the long-chain acyl-CoA hypothesis. *J Biol Chem* 273:16146-16154, 1998
28. Mulder H, Lu D, Finley Jt, An J, Cohen J, Antinozzi PA, McGarry JD, Newgard CB: Overexpression of a modified human malonyl-CoA decarboxylase blocks the glucose-induced increase in malonyl-CoA level but has no impact on insulin secretion in INS-1-derived (832/13) beta-cells. *J Biol Chem* 276:6479-6484, 2001
29. Detimary P, Van den Berghe G, Henquin JC: Concentration dependence and time course of the effects of glucose on adenine and guanine nucleotides in mouse pancreatic islets. *J Biol Chem* 271:20559-20565, 1996
30. Sato Y, Henquin JC: The K_{ATP} channel-independent pathway of regulation of insulin secretion by glucose: in search of the underlying mechanism. *Diabetes* 47:1713-1721, 1998
31. Shiota C, Larsson O, Shelton KD, Shiota M, Efanov AM, Hoy M, Lindner J, Kooptiwut S, Juntti-Berggren L, Gromada J, Berggren PO, Magnuson MA: Sulfonylurea receptor type 1 knock-out mice have intact feeding-stimulated insulin secretion despite marked impairment in their response to glucose. *J Biol Chem* 277:37176-37183, 2002
32. Sharp GW: The adenylate cyclase-cyclic AMP system in islets of Langerhans and its role in the control of insulin release. *Diabetologia* 16:287-296, 1979
33. Gromada J, Holst JJ, Rorsman P: Cellular regulation of islet hormone secretion by the incretin hormone glucagon-like peptide 1. *Pflugers Arch* 435:583-594, 1998
34. MacDonald PE, El-Kholy W, Riedel MJ, Salapatek AM, Light PE, Wheeler MB: The multiple actions of GLP-1 on the process of glucose-stimulated insulin secretion. *Diabetes* 51 Suppl 3:S434-442, 2002
35. Zawalich WS, Rasmussen H: Control of insulin secretion: a model involving Ca^{2+}, cAMP and diacylglycerol. *Mol Cell Endocrinol* 70:119-137, 1990
36. McClenaghan NH, Flatt PR, Ball AJ: Actions of glucagon-like peptide-1 on K_{ATP} channel-dependent and -independent effects of glucose, sulphonylureas and nateglinide. *J Endocrinol* 190:889-896, 2006
37. Kashima Y, Miki T, Shibasaki T, Ozaki N, Miyazaki M, Yano H, Seino S: Critical role of cAMP-GEFII-Rim2 complex in incretin-potentiated insulin secretion. *J Biol Chem* 276:46046-46053, 2001
38. Holz GG: Epac: A new cAMP-binding protein in support of glucagon-like peptide-1 receptor-mediated signal transduction in the pancreatic beta-cell. *Diabetes* 53:5-13, 2004
39. Holz GG: New insights concerning the glucose-dependent insulin secretagogue action of glucagon-like peptide-1 in pancreatic beta-cells. *Horm Metab Res* 36:787-794, 2004
40. Akiba S, Sato T: Cellular function of calcium-independent phospholipase A_2. *Biol Pharm Bull* 27:1174-1178, 2004
41. Ramanadham S, Gross RW, Han X, Turk J: Inhibition of arachidonate release by secretagogue-stimulated pancreatic islets suppresses both insulin secretion and the rise in beta-cell cytosolic calcium ion concentration. *Biochemistry* 32:337-346, 1993

42. Ramanadham S, Song H, Bao S, Hsu FF, Zhang S, Ma Z, Jin C, Turk J: Islet complex lipids: involvement in the actions of group VIA calcium-independent phospholipase A_2 in beta-cells. *Diabetes* 53 Suppl 1:S179-185, 2004
43. Song K, Zhang X, Zhao C, Ang NT, Ma ZA: Inhibition of Ca^{2+}-independent phospholipase A_2 results in insufficient insulin secretion and impaired glucose tolerance. *Mol Endocrinol* 19:504-515, 2005
44. Chakraborti S: Phospholipase A_2 isoforms: a perspective. *Cell Signal* 15:637-665, 2003
45. Balsinde J, Winstead MV, Dennis EA: Phospholipase A_2 regulation of arachidonic acid mobilization. *FEBS Lett* 531:2-6, 2002
46. Wolf BA, Pasquale SM, Turk J: Free fatty acid accumulation in secretagogue-stimulated pancreatic islets and effects of arachidonate on depolarization-induced insulin secretion. *Biochemistry* 30:6372-6379, 1991
47. Guenifi A, Simonsson E, Karlsson S, Ahren B, Abdel-Halim SM: Carbachol restores insulin release in diabetic GK rat islets by mechanisms largely involving hydrolysis of diacylglycerol and direct interaction with the exocytotic machinery. *Pancreas* 22:164-171, 2001
48. Metz SA: Exogenous arachidonic acid promotes insulin release from intact or permeabilized rat islets by dual mechanisms. Putative activation of Ca^{2+} mobilization and protein kinase C. *Diabetes* 37:1453-1469, 1988
49. Knutson KL, Hoenig M: Arachidonic acid-induced down-regulation of protein kinase C delta in beta-cells. *J Cell Biochem* 62:543-552, 1996
50. Persaud SJ, Muller D, Belin VD, Kitsou-Mylona I, Asare-Anane H, Papadimitriou A, Burns CJ, Huang GC, Amiel SA, Jones PM: The role of arachidonic acid and its metabolites in insulin secretion from human islets of langerhans. *Diabetes* 56:197-203, 2007
51. Efendic S, Efanov AM, Berggren PO, Zaitsev SV: Two generations of insulinotropic imidazoline compounds. *Diabetes* 51 Suppl 3:S448-454, 2002
52. Robertson RP, Porte D, Jr.: Adrenergic modulation of basal insulin secretion in man. *Diabetes* 22:1-8, 1973
53. Efendic S, Cerasi E, Luft R: Effect of phentolamine and preperfusion with glucose on insulin release from the isolated perfused pancreas from fasted and fed rats. *Diabetologia* 11:407-410, 1975
54. Ostenson CG, Pigon J, Doxey JC, Efendic S: Alpha 2-adrenoceptor blockade does not enhance glucose-induced insulin release in normal subjects or patients with noninsulin-dependent diabetes. *J Clin Endocrinol Metab* 67:1054-1059, 1988
55. Ostenson CG, Cattaneo AG, Doxey JC, Efendic S: Alpha-adrenoceptors and insulin release from pancreatic islets of normal and diabetic rats. *Am J Physiol* 257:E439-443, 1989
56. Schulz A, Hasselblatt A: An insulin-releasing property of imidazoline derivatives is not limited to compounds that block alpha-adrenoceptors. *Naunyn Schmiedebergs Arch Pharmacol* 340:321-327, 1989
57. Schulz A, Hasselblatt A: Dual action of clonidine on insulin release: suppression, but stimulation when alpha 2-adrenoceptors are blocked. *Naunyn Schmiedebergs Arch Pharmacol* 340:712-714, 1989
58. Chan SL: Role of alpha 2-adrenoceptors and imidazoline-binding sites in the control of insulin secretion. *Clin Sci (Lond)* 85:671-677, 1993
59. Chan SL, Brown CA, Scarpello KE, Morgan NG: The imidazoline site involved in control of insulin secretion: characteristics that distinguish it from I_1- and I_2-sites. *Br J Pharmacol* 112:1065-1070, 1994
60. Jonas JC, Plant TD, Henquin JC: Imidazoline antagonists of alpha 2-adrenoceptors increase insulin release in vitro by inhibiting ATP-sensitive K^+ channels in pancreatic beta-cells. *Br J Pharmacol* 107:8-14, 1992
61. Proks P, Ashcroft FM: Phentolamine block of K_{ATP} channels is mediated by Kir6.2. *Proc Natl Acad Sci U S A* 94:11716-11720, 1997
62. Chan SL, Dunne MJ, Stillings MR, Morgan NG: The alpha 2-adrenoceptor antagonist efaroxan modulates K_{ATP} channels in insulin-secreting cells. *Eur J Pharmacol* 204:41-48, 1991

63. Rustenbeck I, Herrmann C, Ratzka P, Hasselblatt A: Imidazoline/guanidinium binding sites and their relation to inhibition of K_{ATP} channels in pancreatic B-cells. *Naunyn Schmiedebergs Arch Pharmacol* 356:410-417, 1997
64. Zaitsev SV, Efanov AM, Efanova IB, Larsson O, Ostenson CG, Gold G, Berggren PO, Efendic S: Imidazoline compounds stimulate insulin release by inhibition of K_{ATP} channels and interaction with the exocytotic machinery. *Diabetes* 45:1610-1618, 1996
65. Efanov AM, Zaitsev SV, Efanova IB, Zhu S, Ostenson CG, Berggren PO, Efendic S: Signaling and sites of interaction for RX871024 and sulfonylurea in the stimulation of insulin release. *Am J Physiol* 274:E751-757, 1998
66. Zaitsev SV, Efanov AM, Raap A, Efanova IB, Schloos J, Steckel-Hamann B, Larsson O, Ostenson CG, Berggren PO, Mest HJ, Efendic S: Different modes of action of the imidazoline compound RX871024 in pancreatic beta-cells. Blocking of K^+ channels, mobilization of Ca^{2+} from endoplasmic reticulum, and interaction with exocytotic machinery. *Ann N Y Acad Sci* 881:241-252, 1999
67. Eliasson L, Renstrom E, Ammala C, Berggren PO, Bertorello AM, Bokvist K, Chibalin A, Deeney JT, Flatt PR, Gabel J, Gromada J, Larsson O, Lindstrom P, Rhodes CJ, Rorsman P: PKC-dependent stimulation of exocytosis by sulfonylureas in pancreatic beta cells. *Science* 271:813-815, 1996
68. Chan SL, Monks LK, Gao H, Deaville P, Morgan NG: Identification of the monomeric G-protein, Rhes, as an efaroxan-regulated protein in the pancreatic beta-cell. *Br J Pharmacol* 136:31-36, 2002
69. Olmos G, Ribera J, Garcia-Sevilla JA: Imidazoli(di)ne compounds interact with the phencyclidine site of NMDA receptors in the rat brain. *Eur J Pharmacol* 310:273-276, 1996
70. Chan SL, Pallett AL, Clews J, Ramsden CA, Morgan NG: Evidence that the ability of imidazoline compounds to stimulate insulin secretion is not due to interaction with sigma receptors. *Eur J Pharmacol* 323:241-244, 1997
71. Kashiwagi A, Harano Y, Suzuki M, Kojima H, Harada M, Nishio Y, Shigeta Y: New alpha 2-adrenergic blocker (DG-5128) improves insulin secretion and in vivo glucose disposal in NIDDM patients. *Diabetes* 35:1085-1089, 1986
72. Kawazu S, Suzuki M, Negishi K, Watanabe T, Ishii J: Studies of midaglizole (DG-5128). A new type of oral hypoglycemic drug in healthy subjects. *Diabetes* 36:216-220, 1987
73. Lernmark A: The preparation of, and studies on, free cell suspensions from mouse pancreatic islets. *Diabetologia* 10:431-438, 1974
74. Moede T, Leibiger B, Pour HG, Berggren P, Leibiger IB: Identification of a nuclear localization signal, RRMKWKK, in the homeodomain transcription factor PDX-1. *FEBS Lett* 461:229-234, 1999
75. Leibiger B, Leibiger IB, Moede T, Kemper S, Kulkarni RN, Kahn CR, de Vargas LM, Berggren PO: Selective insulin signaling through A and B insulin receptors regulates transcription of insulin and glucokinase genes in pancreatic beta cells. *Mol Cell* 7:559-570, 2001
76. Simonsson E, Karlsson S, Ahren B: Ca^{2+}-independent phospholipase A_2 contributes to the insulinotropic action of cholecystokinin-8 in rat islets: dissociation from the mechanism of carbachol. *Diabetes* 47:1436-1443, 1998
77. Sharoyko VV, Zaitseva, II, Varsanyi M, Portwood N, Leibiger B, Leibiger I, Berggren PO, Efendic S, Zaitsev SV: Monomeric G-protein, Rhes, is not an imidazoline-regulated protein in pancreatic beta-cells. *Biochem Biophys Res Commun* 338:1455-1459, 2005
78. Chan SL, Mourtada M, Morgan NG: Characterization of a K_{ATP} channel-independent pathway involved in potentiation of insulin secretion by efaroxan. *Diabetes* 50:340-347, 2001
79. Rustenbeck I, Wienbergen A, Bleck C, Jorns A: Desensitization of insulin secretion by depolarizing insulin secretagogues. *Diabetes* 53 Suppl 3:S140-150, 2004
80. Davalli AM, Pontiroli AE, Socci C, Bertuzzi F, Fattor B, Braghi S, Di Carlo V, Pozza G: Human islets chronically exposed in vitro to different stimuli become unresponsive to the same stimuli given acutely: evidence supporting specific desensitization rather than beta-cell exhaustion. *J Clin Endocrinol Metab* 74:790-794, 1992

81. Gullo D, Rabuazzo AM, Vetri M, Gatta C, Vinci C, Buscema M, Vigneri R, Purrello F: Chronic exposure to glibenclamide impairs insulin secretion in isolated rat pancreatic islets. *J Endocrinol Invest* 14:287-291, 1991
82. Rabuazzo AM, Buscema M, Vinci C, Caltabiano V, Vetri M, Forte F, Vigneri R, Purrello F: Glyburide and tolbutamide induce desensitization of insulin release in rat pancreatic islets by different mechanisms. *Endocrinology* 131:1815-1820, 1992
83. Jagerbrink T, Lexander H, Palmberg C, Shafqat J, Sharoyko V, Berggren PO, Efendic S, Zaitsev S, Jornvall H: Differential protein expression in pancreatic islets after treatment with an imidazoline compound. *Cell Mol Life Sci* 64:1310-1316, 2007
84. Zerangue N, Schwappach B, Jan YN, Jan LY: A new ER trafficking signal regulates the subunit stoichiometry of plasma membrane K_{ATP} channels. *Neuron* 22:537-548, 1999
85. Seghers V, Nakazaki M, DeMayo F, Aguilar-Bryan L, Bryan J: Sur1 knockout mice. A model for K_{ATP} channel-independent regulation of insulin secretion. *J Biol Chem* 275:9270-9277, 2000
86. Separovic D, Kester M, Haxhiu MA, Ernsberger P: Activation of phosphatidylcholine-selective phospholipase C by I_1-imidazoline receptors in PC12 cells and rostral ventrolateral medulla. *Brain Res* 749:335-339, 1997
87. Separovic D, Kester M, Ernsberger P: Coupling of I_1-imidazoline receptors to diacylglyceride accumulation in PC12 rat pheochromocytoma cells. *Mol Pharmacol* 49:668-675, 1996
88. Munk SA, Lai RK, Burke JE, Arasasingham PN, Kharlamb AB, Manlapaz CA, Padillo EU, Wijono MK, Hasson DW, Wheeler LA, Garst ME: Synthesis and pharmacologic evaluation of 2-endo-amino-3-exo-isopropylbicyclo[2.2.1]heptane: a potent imidazoline1 receptor specific agent. *J Med Chem* 39:1193-1195, 1996
89. Efanov AM, Zaitsev SV, Berggren PO, Mest HJ, Efendic S: Imidazoline RX871024 raises diacylglycerol levels in rat pancreatic islets. *Biochem Biophys Res Commun* 281:1070-1073, 2001
90. Falk JD, Vargiu P, Foye PE, Usui H, Perez J, Danielson PE, Lerner DL, Bernal J, Sutcliffe JG: Rhes: A striatal-specific Ras homolog related to Dexras1. *J Neurosci Res* 57:782-788, 1999
91. Seino S, Shibasaki T: PKA-dependent and PKA-independent pathways for cAMP-regulated exocytosis. *Physiol Rev* 85:1303-1342, 2005
92. Doyle ME, Egan JM: Mechanisms of action of glucagon-like peptide 1 in the pancreas. *Pharmacol Ther* 113:546-593, 2007
93. Nowatzke W, Ramanadham S, Ma Z, Hsu FF, Bohrer A, Turk J: Mass spectrometric evidence that agents that cause loss of Ca^{2+} from intracellular compartments induce hydrolysis of arachidonic acid from pancreatic islet membrane phospholipids by a mechanism that does not require a rise in cytosolic Ca^{2+} concentration. *Endocrinology* 139:4073-4085, 1998
94. Needleman P, Turk J, Jakschik BA, Morrison AR, Lefkowith JB: Arachidonic acid metabolism. *Annu Rev Biochem* 55:69-102, 1986
95. Jones PM, Persaud SJ: Arachidonic acid as a second messenger in glucose-induced insulin secretion from pancreatic beta-cells. *J Endocrinol* 137:7-14, 1993
96. Ramanadham S, Song H, Hsu FF, Zhang S, Crankshaw M, Grant GA, Newgard CB, Bao S, Ma Z, Turk J: Pancreatic islets and insulinoma cells express a novel isoform of group VIA phospholipase A_2 ($iPLA_2$ beta) that participates in glucose-stimulated insulin secretion and is not produced by alternate splicing of the $iPLA_2$ beta transcript. *Biochemistry* 42:13929-13940, 2003
97. Efanov AM, Appelskog IB, Abdel-Halim SM, Khan A, Branstrom R, Larsson O, Ostenson CG, Mest HJ, Berggren PO, Efendic S, Zaitsev SV: Insulinotropic activity of the imidazoline derivative RX871024 in the diabetic GK rat. *Am J Physiol Endocrinol Metab* 282:E117-124, 2002
98. Falck JR, Manna S, Moltz J, Chacos N, Capdevila J: Epoxyeicosatrienoic acids stimulate glucagon and insulin release from isolated rat pancreatic islets. *Biochem Biophys Res Commun* 114:743-749, 1983
99. Woodcroft KJ, Szczepan EW, Knickle LC, Bend JR: Three N-aralkylated derivatives of 1-aminobenzotriazole as potent and isozyme-selective, mechanism-based inhibitors of guinea pig pulmonary cytochrome P-450 in vitro. *Drug Metab Dispos* 18:1031-1037, 1990