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Research Article

Arachidonic acid signaling is involved in the mechanism of imidazoline-induced K_{ATP} channel-independent stimulation of insulin secretion

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Abstract. The mechanism by which the novel, pure glucose-dependent insulinotropic, imidazoline derivative BL11282 promotes insulin secretion in pancreatic islets has been investigated. The roles of K_{ATP} channels, α_2-adrenoreceptors, the I_1-receptor-phosphatidylcholine-specific phospholipase (PC-PLC) pathway and arachidonic acid signaling in BL11282 potentiation of insulin secretion in pancreatic islets were studied. Using $SUR1^{(-/-)}$ deficient mice, the previous notion that the insulinotropic activity of BL11282 is not related to its interaction with K_{ATP} channels was confirmed. Insulinotropic activity of BL11282 was not related to its effect on α_2-adrenoreceptors, I_1-imidazoline receptors or PC-PLC. BL11282 significantly increased [^{3}H]arachidonic acid production. This effect was abolished in the presence of the $iPLA_2$ inhibitor, bromoenol lactone. The data suggest that potentiation of glucose-induced insulin release by BL11282, which is independent of concomitant changes in cytoplasmic free Ca^{2+} concentration, involves release of arachidonic acid by $iPLA_2$ and its metabolism to epoxyeicosatrienoic acids through the cytochrome P-450 pathway.

Keywords. BL11282, imidazolines, insulin secretion, arachidonic acid, calcium-independent phospholipase A_2, cytochrome P-450.

Introduction

Many compounds with an imidazoline moiety are effective stimulators of insulin secretion in pancreatic β-cells [1–5]. It was proposed that the insulinotropic effect of these compounds involves blockade of K_{ATP} channels [6] and specifically the Kir6.2 subunit of this channel [7]. A decade ago, we defined a K_{ATP} channel-independent pathway by which imidazoline compounds stimulate insulin secretion [8]. We demonstrated that the imidazoline compound RX871024 promotes insulin release by two modes of action [8–10]. One mode includes the blockade of K_{ATP} channels, membrane depolarization and activation of voltage-dependent Ca^{2+} channels. Another is associated with distal components of the exocytotic pathway and is not related to changes in membrane potential and $[Ca^{2+}]_i$ [8–10]. Having effects on K_{ATP} channel activity, RX871024, like sulfonylureas, stimulated

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insulin secretion even at low glucose concentration [8–10]. The strong insulinotropic effect of these compounds observed at low glucose concentrations is not desirable because it can provoke pronounced hypoglycemia. Therefore, we developed a novel, pure glucose-dependent, insulinotropic imidazoline compound, BL11282, which directly affects the insulin exocytotic machinery and does not block K_{ATP} channel activity [11, 12]. BL11282 does not induce insulin secretion at basal glucose concentration, whereas it stimulates insulin secretion at elevated glucose level [11, 12]. Such imidazoline compounds are potentially new therapeutic agents for treatment of type 2 diabetes [12]. However, so far, the detailed biochemical and pharmacological mechanisms underlying the K_{ATP} channel-independent effect of BL11282 on insulin release remain unknown.

The aim of this study was to investigate the signal-transduction pathway involved in the insulinotropic action of BL11282. In pancreatic islets, in addition to closure of K_{ATP} channels, imidazoline compounds were shown to affect α_2-adrenoreceptors [5, 6], imidazoline receptors (especially the thoroughly investigated I_1-receptor) and also arachidonic acid signaling [12]. We therefore studied the role of α_2-adrenoreceptors, I_1-receptor-phosphatidylcholine phospholipase-specific (PC-PLC) pathway and arachidonic acid signaling in BL11282-potentiated insulin secretion in pancreatic islets. We unambiguously confirmed the K_{ATP} channel-independent insulinotropic mechanism of BL11282 action using $SUR1^{(-/-)}$ mice.

Materials and methods

Materials. BL11282 [5-chloro-3-(4,5-dihydro-1H-imidazol-2-yl)-2-methylindole hydrochloride] was obtained from Eli Lilly (Indianapolis, IN, USA). Diazoxide (7-chloro-3-methyl-4H-1,2,4-benzothiadiazine-1,1-dioxide), BEL [bromoenol lactone; 2H-Pyran-2-one, 6-(bromoethylene)tetrahydro-3-(1-naphthalenyl)-, (E)-], MB-1-ABT (*N*-α-methylbenzyl-1-aminobenzotriazole), AGN192403 (2-*endo*-amino-3-exo-isopropylbicyclo[2.2.1]heptane hydrochloride) and yohimbine (17-hydroxyyohimban-16-carboxylic acid methyl ester hydrochloride) 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-ylmethylisobutylamino)-1-[2-(2,4-difluorobenzoyl)-benzoyl]-pyrrolidin-2-ylmethyl}-3-[4-(2,4-dioxithiazolidin-5-ylidenemethyl)-phenyl]acrylamide hydrochloride) were purchased from Calbiochem (San Diego, CA, USA). 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.

Isolation of rat pancreatic islets. The experiments were approved by the ethical committee of Karolinska Institutet (Stockholm, Sweden). Non-diabetic 2–3-month-old Wistar rats were obtained from B&K Universal (Sollentuna, Sweden). Diabetic 2–3-month-old Goto-Kakizaki (GK) rats 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, TN, USA) [13]. Islets from mice and rats were isolated by collagenase digestion as previously described [14, 15]. Isolated pancreatic islets were incubated overnight 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.

Measurements of insulin secretion. Insulin secretion from islets was measured in Krebs-Ringer bicarbonate buffer (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 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. For static measurements, 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 substances. To measure insulin secretion in islets under depolarized conditions, KRBB containing 55 mM KCl, 250 µM diazoxide and 3.3 or 16.7 mM glucose was used. The incubation medium was removed and stored at –20 °C until its insulin content was analyzed by radioimmunoassay, employing rat insulin as standard.

RNA extraction and semi-quantitative RT-PCR. Islets were collected under a stereomicroscope 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. RNA samples were stored at –80 °C. Reverse transcription was carried out using Super-Script II First-Strand Synthesis System (Invitrogen–Life Technologies, CA, USA) according to the man-

ufacturer'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) in a final volume of 20 μl as described [15]. 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 within 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 ribosomal protein L30 (rPL30) and iPLA$_2$β fragments were within the linear range. This was verified by testing various numbers of amplification cycles (cycles from 23 to 27 and cycles from 29 to 33 for rPL30 and iPLA$_2$β, respectively). 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 ng total RNA. The specific primers for rPL30 [15] for rat islets were used as internal controls. The specific primers used for iPLA$_2$β cDNA (RGD: 628867) were forward 5′-CAGAGAATGAGGAGGGCTGT-3′ and reverse 5′-GGATCCTTGCTGTGGATCTG-3′. 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). The predicted PCR product sizes were 228 bp for rPL30 and 412 bp for iPLA$_2$β. All PCRs included reverse transcription-negative controls, and these reactions consistently yielded no amplification product. PCR products were gel-purified (Nucleo-Trap, Clontech, Germany) and cloned with a TOPO-TA cloning kit (Invitrogen-Life Technologies), as recommended by the manufacturer.

Measurements of arachidonic acid release from pancreatic islets. Generation of arachidonic acid was quantified by efflux of [^{3}H]arachidonic acid from [^{3}H]arachidonic acid-prelabeled islets, as previously described [16, 17]. 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 the 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.

Statistical analysis. 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 ANOVA followed by the LSD test. Differences between mean values were considered significant if $p < 0.05$.

Results

Imidazoline compound BL11282 does not interact with K_{ATP} channels. It has previously been shown that the insulinotropic properties of imidazoline compound BL11282 were not related to activation of K_{ATP} channels [11]. For additional verification of this fact we examined a mouse model with a deletion of the regulatory subunit, SUR1, of K_{ATP} channels [13]. Insulin secretion measurements were done in isolated pancreatic islets from control and SUR1$^{(-/-)}$ mice. At 3.3 mM glucose, insulin release from SUR1$^{(-/-)}$ mouse islets was significantly higher than from control NMRI mouse islets (Fig. 1). At 16.7 mM glucose, 10 μM BL11282 potentiated insulin secretion in islets from both control and SUR1$^{(-/-)}$ mice (Fig. 1).

α_2-adrenoreceptors do not mediate insulinotropic action of BL11282. To investigate whether the insulinotropic action of imidazoline compound BL11282 could be explained by α_2-adrenergic antagonistic activity, experiments were done in the presence of the α_2-adrenergic selective competitive antagonist yohimbine (Fig. 2a). The pancreatic islets were incubated with 10 μM yohimbine alone or in the presence of 50 μM BL11282 (Fig. 2a). The antagonist did not affect insulin release at 3.3 or 16.7 mM glucose when given alone or in combination with BL11282.

Imidazoline I_1-receptors and the PC-PLC pathway are not involved in the insulinotropic action of BL11282. The functional role of I_1-receptors in

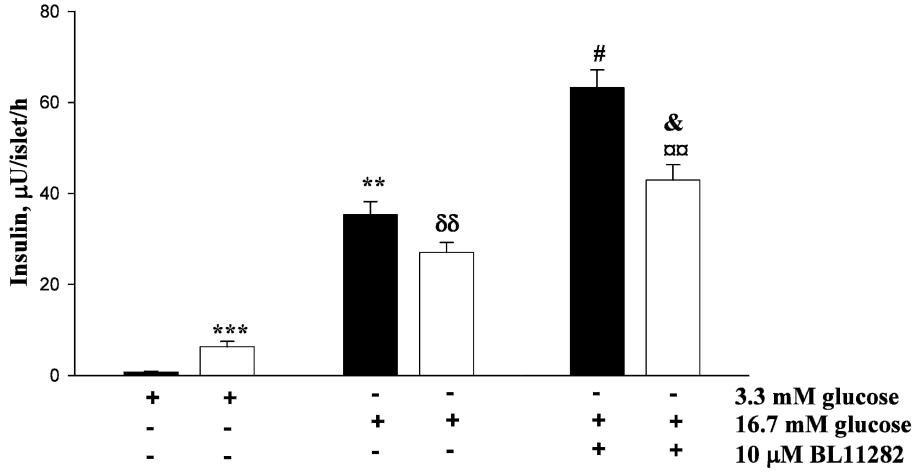

Figure 1. BL11282 stimulates glucose-induced insulin secretion in SUR1$^{(-/-)}$ mouse islets. BL11282 (10 μM) was added to isolated pancreatic islets from NMRI (black columns) and SUR1$^{(-/-)}$ (white columns) mice. Data are presented as means ± SE for three independent experiments [$^{**}p < 0.01$, $^{***}p < 0.001$ relative to 3.3 mM glucose, NMRI mice; $^{\delta\delta}p < 0.01$ relative to 3.3 mM glucose, SUR1$^{(-/-)}$ mice; $^{\#}p < 0.05$ relative to 16.7 mM glucose, NMRI mice; $^{\&}p < 0.05$ relative to 16.7 mM glucose + BL11282, NMRI mice; $^{\infty}p < 0.01$ relative to 16.7 mM glucose, SUR1$^{(-/-)}$ mice].

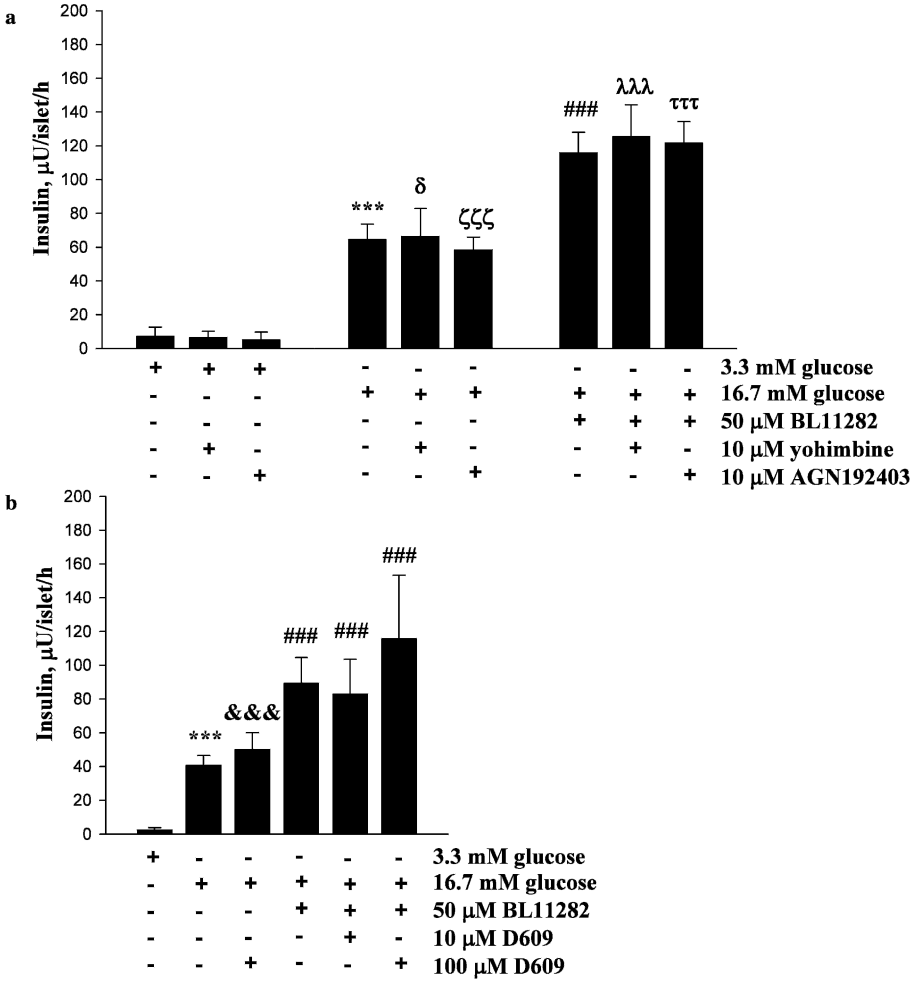

Figure 2. Role of α_2-adrenoreceptors and imidazoline I_1-receptors in the insulinotropic action of BL11282. (*a*) Effect of yohimbine and AGN192403 on insulin secretion induced by 50 μM BL11282 in isolated pancreatic islets from Wistar rats. Data are presented as means ± SE for three independent experiments. (*b*) Effect of D609 on insulin secretion induced by 50 μM BL11282 in isolated pancreatic islets from Wistar rats. Data are presented as means ± SE for four independent experiments ($^{***}p < 0.001$ relative to 3.3 mM glucose; $^{\delta}p < 0.05$ relative to 3.3 mM glucose + yohimbine; $^{\zeta\zeta\zeta}p < 0.001$ relative to 3.3 mM glucose + AGN192403; $^{\#\#\#}p < 0.001$ relative to 16.7 mM glucose; $^{\lambda\lambda\lambda}p < 0.001$ relative to 16.7 mM glucose + yohimbine; $^{\tau\tau\tau}p < 0.001$ relative to 16.7 mM glucose + AGN192403; $^{\&\&\&}p < 0.001$ relative to 16.7 mM glucose + BL11282).

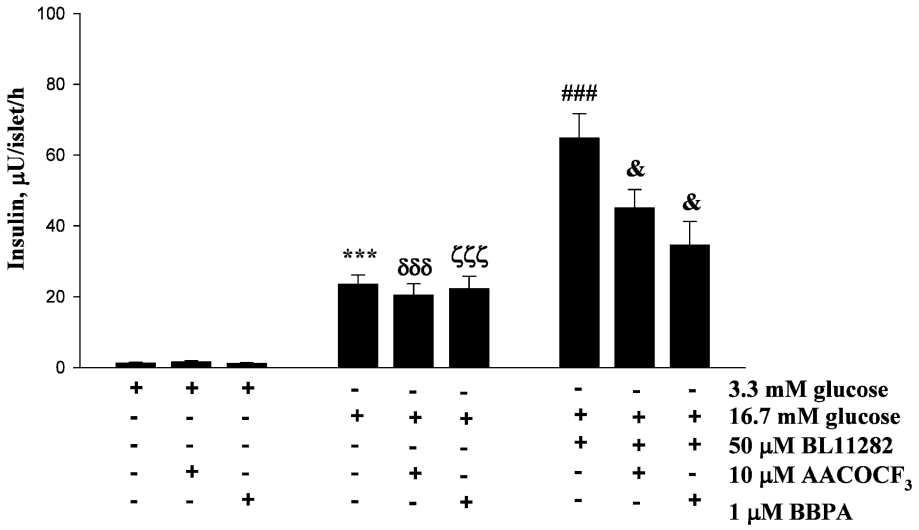

Figure 3. $AACOCF_3$ and BBPA, $cPLA_2$ inhibitors, partially suppress BL11282-stimulated insulin secretion in isolated pancreatic islets from Wistar rats. Data are presented as means ± SE for six independent experiments (***$p < 0.001$ relative to 3.3 mM glucose; $^{\delta\delta\delta}p < 0.001$ relative to 3.3 mM glucose + $AACOCF_3$; $^{\zeta\zeta\zeta}p < 0.001$ relative to 3.3 mM glucose + BBPA; $^{\#\#\#}p < 0.001$ relative to 16.7 mM glucose; $^{\&}p < 0.05$ relative to 16.7 mM glucose + 50 μM BL11282).

BL11282-dependent insulin secretion was studied using a selective I_1-receptor antagonist AGN192403 [18]. The data depicted in Figure 2a show that incubation of pancreatic islets with 10 μM AGN192403 did not affect insulin secretion at 3.3 or 16.7 mM glucose as well as BL11282-stimulated insulin release. In addition, we investigated the involvement of PC-PLC, the enzyme that has been shown to be coupled to the I_1-receptor, using the inhibitor of PC-PLC, D-609 [19]. The data presented in Figure 2b show that 10 and 100 μM D609 did not modulate the effect of BL11282 on insulin release.

The role of Ca^{2+}-dependent phospholipase A_2 ($cPLA_2$) in the insulinotropic activity of BL11282. To investigate the role of $cPLA_2$ in BL11282-dependent insulin secretion we employed two selective inhibitors of $cPLA_2$: $AACOCF_3$ and BBPA. The data depicted in Figure 3 show that both 10 μM $AACOCF_3$ and 1 μM BBPA do not inhibit insulin secretion in islets at basal and stimulatory glucose concentrations. In the presence of a high glucose concentration and BL11282, there was a partial suppression of insulin secretion by $AACOCF_3$ and BBPA (Fig. 3). However, at 16.7 mM glucose, under depolarized conditions (55 mM KCl and 250 μM diazoxide) when $[Ca^{2+}]_i$ is clamped [20], there was no effect of BBPA on BL11282-stimulated insulin secretion. Insulin release was 263 ± 38 μU/islet/h and 251 ± 35 μU/islet/h with and without BL11282, respectively.

Ca^{2+}-independent phospholipase A_2 ($iPLA_2$) is involved in the insulinotropic action of BL11282. There is evidence that inhibition of $iPLA_2$ in mouse pancreatic islets decreases glucose-stimulated insulin secretion [21]. We compared $iPLA_2\beta$ isoform expression levels between normal Wistar rat and diabetic GK rat islets. The data in Figure 4a demonstrate that $iPLA_2\beta$ mRNA expression level decreased in GK rat islets compared to Wistar rat islets.

We next investigated the role of $iPLA_2$ in the effects of BL11282 on insulin release independent of concomitant changes in $[Ca^{2+}]_i$. For this study we used an inhibitor of $iPLA_2$, BEL [22, 23], and performed experiments under depolarized conditions. Results presented in Figure 4b demonstrate that under these conditions BEL decreased insulin release at 16.7 mM glucose. In addition, we observed that the effect of BL11282 on insulin secretion in the presence of BEL was completely abolished. Under depolarized conditions at basal glucose concentration (3.3 mM) BEL inhibited insulin release.

For further verification of the role of $iPLA_2$ in the insulinotropic activity of BL11282, measurements of [^{3}H]arachidonic acid release from the islets in the presence and absence of the imidazoline and BEL were performed. The results in Figure 5 demonstrate that increased glucose concentration did not significantly affect [^{3}H]arachidonic acid release. However, in the presence of high glucose concentration BL11282 significantly stimulated [^{3}H]arachidonic acid release. This effect was abolished in the presence of the $iPLA_2$ inhibitor BEL.

Cytochrome P-450 mediates the insulinotropic action of BL11282. Epoxyeicosatrienoic acids generated by cytochrome P-450 from arachidonic acid have been shown to stimulate insulin secretion [24]. To elucidate the role of cytochrome P-450 in BL11282-potentiated insulin secretion, we have used MB-1-ABT, a selective inhibitor of cytochrome P-450 [25]; 200 μM MB-1-

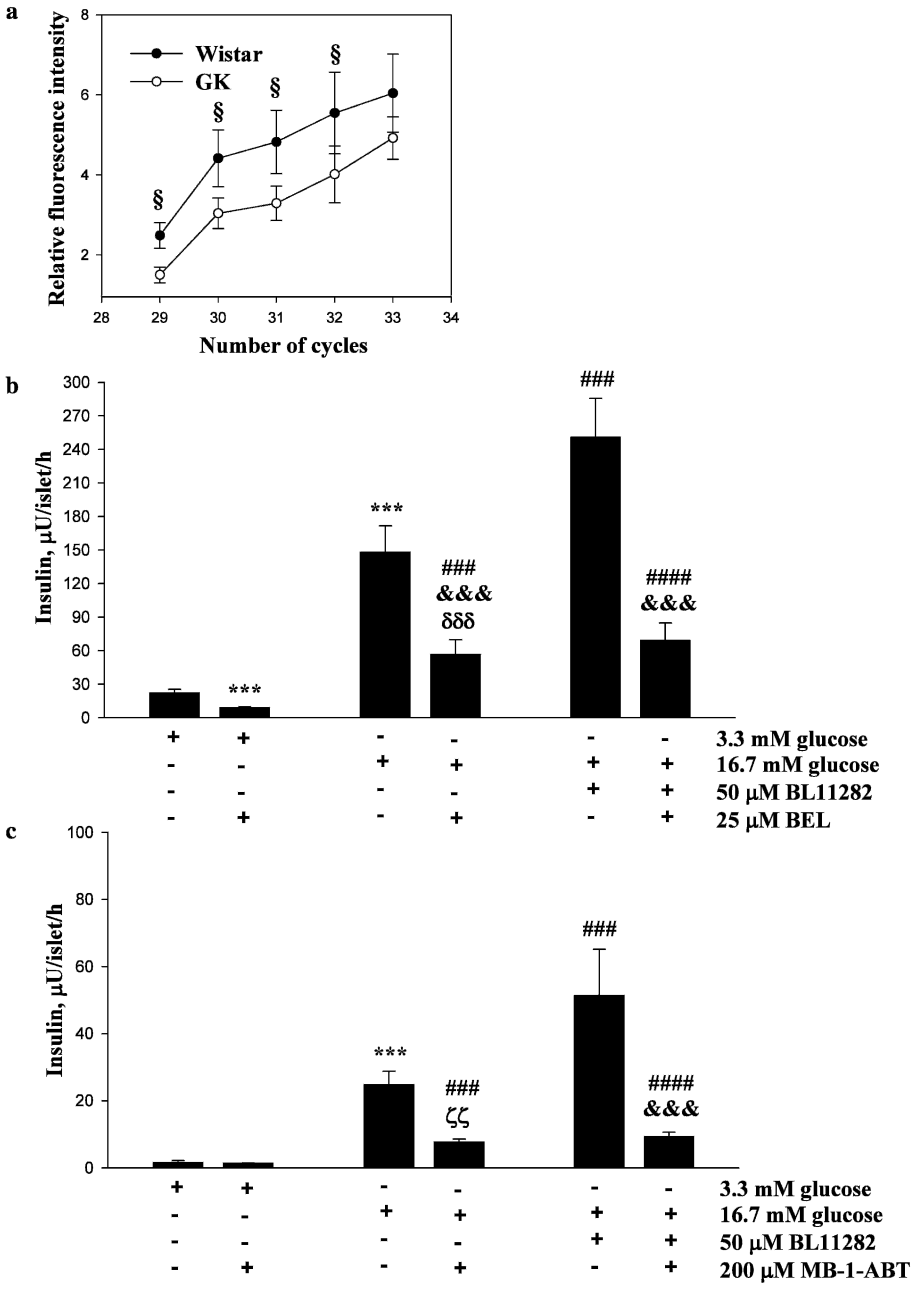

Figure 4. Role of $iPLA_2$ and cytochrome P-450 in the insulinotropic action of BL11282. (*a*) Semi-quantitative RT-PCR analysis of relative $iPLA_2\beta$ mRNA expression in Wistar rat and GK rat pancreatic islets. Data are from four independent pools of total mRNA from freshly isolated islets. Normalization was performed for rPL30. (*b*) Effect of the $iPLA_2$ inhibitor, BEL, on insulin secretion induced by 50 μM BL11282 under depolarized conditions in isolated pancreatic islets from Wistar rats. Data are presented as means ± SE for six independent experiments. (*c*) Effect of the cytochrome P-450 inhibitor, MB-1-ABT, on insulin secretion induced by 50 μM BL11282 in isolated pancreatic islets from Wistar rats. Data are presented as means ± SE for three independent experiments. ($^{\S}p < 0.05$ relative to GK rat pancreatic islets; $^{***}p < 0.001$ relative to 3.3 mM glucose; $^{\delta\delta\delta}p < 0.001$ relative to 3.3 mM glucose + BEL; $^{\#\#\#}p < 0.001$ relative to 16.7 mM glucose; $^{\zeta\zeta}p < 0.01$ relative to 3.3 mM glucose + MB-1-ABT; $^{\&\&\&}p < 0.001$ relative to 16.7 mM glucose + 50 μM BL11282).

ABT inhibited glucose-induced insulin release and fully suppressed BL11282-stimulated insulin secretion (Fig. 4c). The inhibitor MB-1-ABT did not influence insulin secretion at 3.3 mM glucose.

Discussion

We have previously reported that the imidazoline compound BL11282 stimulates insulin secretion without blockade of K_{ATP} channels [11, 12]. We confirmed this observation in the $SUR1^{(-/-)}$ knockout mouse model. Removing the SUR1 subunits blocks the assembly of Kir6.2 subunits into a functionally active channel and transport from the ER [26, 27]. Hence, insulin release in $SUR1^{(-/-)}$ islets stimulated by BL11282 cannot be attributed to the interaction of BL11282 with K_{ATP} channels. As K_{ATP} channels determine membrane potential in pancreatic β-cells, cells without functional K_{ATP} channels demonstrate constant depolarization and increased concentration of $[Ca^{2+}]_i$, independently from extracellular glucose concentration [27]. Therefore, at 3.3 mM glucose, we observed higher insulin release in islets from $SUR1^{(-/-)}$ knockout mice compared to control wild-type islets. BL11282 potentiated insulin secre-

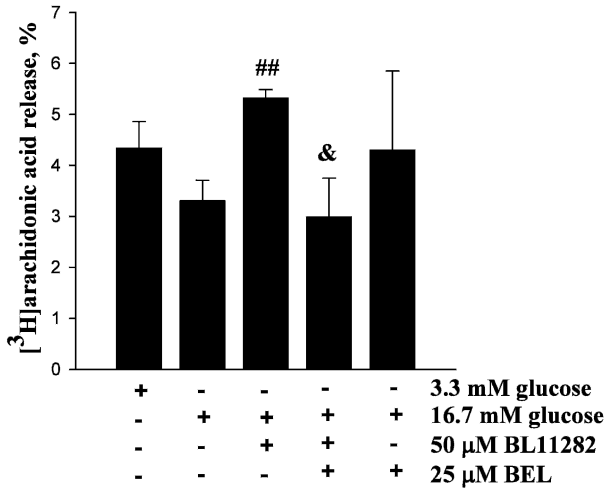

Figure 5. BEL, $iPLA_2$ inhibitor, blocks BL11282-stimulated [^{3}H]arachidonic acid release in isolated pancreatic islets from Wistar rats. After treatment, the incubation buffer was removed and radioactivity was determined in the removed buffer and islets by liquid scintillation counting. [^{3}H]Arachidonic acid release was estimated as the percentage of radioactivity in the removed buffer relative to total islet radioactivity ($^{\#\#}p < 0.01$ relative to 16.7 mM glucose; $^{\&}p < 0.05$ relative to 16.7 mM glucose + BL11282).

tion 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 [1, 2]. To evaluate whether BL11282 possesses the α_2-adrenergetic activity, we used the α_2-adrenergic antagonist yohimbine. 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 [28, 29]. 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 [29]. An inhibitor of PC-PLC, D609, blocked moxonidine effect on DAG concentration [29]. To examine whether imidazoline BL11282 is involved in a signaling pathway coupled to I_1-receptor/PC-PLC, we used a selective I_1-receptor antagonist AGN192403 [18] and PC-PLC inhibitor D609 [19]. 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.

BL11282 was demonstrated to stimulate insulin exocytosis in islets at steps distal to the rise in $[Ca^{2+}]_i$ [11, 12]. 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 $[Ca^{2+}]_i$ [30]. These pathways include either intact arachidonic acid or its biologically active metabolites generated by cytochrome P-450, leading to epoxyeicosatrienoic acids [31, 32]. In pancreatic islets arachidonic acid is released from phospholipids (mediated by PLA_2s activity) [33, 34]. To evaluate the role of these pathways in BL11282-stimulated insulin secretion we used the inhibitors of these enzymes.

Based on Ca^{2+} requirements needed for basal activity, intracellular PLA_2s can be divided into two groups: $cPLA_2$ ($[Ca^{2+}]_i$-dependent, group IV) and $iPLA_2$ ($[Ca^{2+}]_i$-independent, group VI). $cPLA_2$ requires micromolar concentrations of $[Ca^{2+}]_i$ for membrane translocation but not for catalysis and possesses a selectivity towards phospholipids containing arachidonic acid moiety [33, 34]. $iPLA_2$ exhibits an absence of substrate specificity for arachidonic acid-containing phospholipids and no $[Ca^{2+}]_i$ requirement for activity [33, 34]. We evaluated the effects of $cPLA_2$ and $iPLA_2$ inhibitors on BL11282-stimulated insulin release under normal and under 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, these 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 [35]. 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 [36]. Therefore, these findings support the idea that $iPLA_2\beta$ is an important player in insulin secretion and that 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 [12]. The results with the use of BEL, 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 (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 (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. 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 [24]. In our previous work, we demonstrated the suppressive effect of the cytochrome P-450 inhibitor MB-1-ABT [25] on insulin secretion induced by glucose and imidazoline compound RX871024 [12]. We have evaluated the effect of the cytochrome P-450 inhibitor MB-1-ABT [25] 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 and in the presence of BL11282 (Fig. 4c). These observations suggest that arachidonic acid metabolism by cytochrome P-450, leading to epoxyeicosatrienoic acids, is important in the potentiation of glucose-induced insulin release by the imidazoline compound BL11282.

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. Our data 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.

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