The Possible Mechanisms by which Borapetol B Stimulates Insulin Release from Rat Islets

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ABSTRACT

We have recently demonstrated the anti-diabetic effect of borapetol B (C1) isolated from the plant *Tinospora crispa* to be associated with stimulation of insulin release in the pancreatic islets. This present study aims to reveal the mechanisms by which C1 stimulates insulin release in the isolated pancreatic islets of normoglycemic control Wistar (W) and spontaneously type 2 diabetic Goto-Kakizaki (GK) rats. Isolated pancreatic islets from Wistar (W) and Goto Kakizaki (GK) rat were cultured overnight and then exposed to incubation conditions at 3.3 mM and 16.7 mM glucose. Several modulators and inhibitors were used; 0.25 mM diazoxide only (to open the K-ATP channel, 50 mM of KCl (for depolarization of beta cells), nifedipine (to block the L-type Ca^{2+} channels), 10 uM H89 and 1.5 uM of calphostin-C (to block PKA-and PKC respectively) and 100 ng/ml pertussis toxin (to inhibit the G_e protein).

The insulin released during incubation was measured using radioimmunoassay (RIA) assay. C1 significantly stimulated insulin release at both low and high glucose in W and GK rat islets. The opening of K-ATP channels by adding diazoxide inhibited insulin release at 16.7 mM glucose in W (P<0.01) and GK (P<0.05) rat islets compared to control. Diazoxide decreased insulin response to C1 in W and in GK (both P<0.01) only at 16.7 mM glucose. The insulin release of both W and GK rat islets incubated with C1+diazoxide+KCl was significantly higher (when compared with islets incubated either with C1 or only or diazoxide+KCl) at both 3.3 mM and 16.7 mM glucose. Nifedipine decreased insulin release in W (P<0.05) and in GK (P<0.01) rat islets at 16.7 glucose only. In the presence of nifedipine, C1-induced insulin secretion of islets was decreased in W (P<0.01) and GK rat islets at 16.7 mM glucose. H89 and calphostin C inhibitors did not affect the insulin response to C1 respectively in W and GK islets at both 3.3 mM and 16.7 mM glucose. At 16.7 mM glucose, pertussis toxin decreased the insulin response to C1 in the W (P<0.01) and GK rat islets (P<0.05). When exploring the mechanisms of insulin release in the W and GK pancreatic islets, we showed that the C1 effect was exerted partly via K-ATP channels since diazoxide partly, but not totally suppressed C1 stimulation at 16.7 mM glucose. C1 effect was also dependent on L-type Ca^{2+} channels since nifedipine suppressed the insulin response to C1 at 16.7 mM. There was no modulation by PKA and PKC inhibitors. Furthermore, C1 effect was partly dependent on pertussis toxin sensitive G_e-protein. Therefore, the major stimulatory effect of C1 might be on the exocytosis.

Keywords-Type 2 diabetes, *Tinospora crispa*, insulin secretion, Goto kakizaki rat, pancreatic islets

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1. INTRODUCTION

Impaired insulin secretion in type 2 diabetes (T2D) is caused by beta-cell secretory dysfunction and/or decreased beta-cell mass which results in the failure of islets to secrete adequate insulin in order to achieve normoglycaemia [1].

The K-ATP channel-dependent and K-ATP channel-independent pathways are two major signaling pathways involved in controlling the insulin secretion and maintaining the regulation of glucose homeostasis in the pancreatic islets [2,3]. In the K-ATP dependent pathway, glucose enters the beta cells via GLUT-2 transporter and its metabolism leads to the increase in the cellular adenosine triphosphate/adenosine diphosphate (ATP/ADP) ratio. This leads to the closure of ATP-dependent K^+ channels causing membrane depolarization, opening of voltage dependent Ca^{2+} channels, that facilitates free extracellular Ca^{2+} to enter the cells, causing increased cytosolic Ca^{2+} concentration and stimulated insulin release by exocytosis [4-7]. The K-ATP channel-independent pathway, in which glucose exerts direct stimulatory effects on the exocytosis of insulin, works in synergy with the K-ATP channel-dependent pathway [8-9]. Second messengers such as cyclic adenosine monophosphate (cAMP) and diacylglycerol (DAG) increase insulin release via PKA and PKC pathways [10]. The guanyl-nucleotide-binding (GTP coupled) proteins, also known as G proteins are involved in the process of exocytosis [11-12].

We recently showed that borapetol B (C1), isolated from the plant *Tinospora crispa*, decreased the blood glucose levels and increased serum insulin levels in control Wistar (W) and diabetic Goto-Kakizaki (GK) rats [13]. The blood glucose lowering-effect was found be associated with the stimulation of insulin release by C1 in a dose-dependent manner. In this study, we aimed to identify the mechanism by which C1 stimulates insulin response in the pancreatic islets using normoglycemic control W and diabetic GK rats, an animal model of type 2diabetes [14].

2. MATERIALS AND METHODS

2.1 Animals

Male normoglycemic control Wistar (W) and spontaneously type 2 diabetic Goto-Kakizaki (GK) rats (200-350 g) were used in this study. GK rats, originating from W rats, were bred in our department [14]. W rats were purchased from a commercial breeder (Charles River). The animals were kept at 22°C with an alternating 12-hour light-dark cycle (6 am-6 pm) and were allowed access to food and water before being anesthetized for isolation of pancreatic islets. The study was approved by the Laboratory Animal Ethics Committee of the Karolinska Institutet.

2.2 Preparation of C1 and Isolation of Pancreatic Islets

Fractionation and purification of borapetol B (C1) from *T.crispa* was done based on the method described previously [13, 15]. The isolation of W and GK islets was performed using collagenase digestion method by injecting Hank's Balanced Salt Solution (HBBS) (StatensVeterinärAnstalt, Sweden) containing collagenase (Sigma-Aldrich, USA) into the bile duct. The amount of collagenase added was 9 mg and 24 mg for W and GK rats, respectively, diluted in 10 ml of HBBS. The isolated pancreas was incubated in 37°C water bath without shaking for 24 minutes, followed by several washing and centrifugation steps with HBBS, Histopaque 1119 (Sigma-Aldrich, USA) and Histopaque 1077 (Sigma-Aldrich, USA). The islets were hand-picked under a stereomicroscope and then cultured for 24 hours at 37°C, with an atmosphere of 5% CO_2-95% air in RPMI 1640 culture medium (SVA, Sweden) supplemented with 30 mg L-glutamine (Sigma-Aldrich, USA), 11 mM glucose (Sigma-Aldrich, USA), antibiotics (100 IU/ml penicillin and 0.1 mg/ml streptomycin) (Invitrogen, USA). Inactivated fetal calf serum (10 %) was added to RPMI 1640 medium (SVA, Sweden) before the incubation of islets [14]. Following the overnight incubation, the islets were transferred in 3.3 mM glucose and incubated at 37°C for 60 min before being used for further experiments.

2.3 Stimulation of Insulin Release by C1

To assess the effect of C1 on the K-ATP channel, several inhibitors were used to inhibit the insulin release (Figure 1). W and GK rat islets supplemented with 0.25 mM diazoxide (to open the K-ATP channels) and 50 mM of KCl (for depolarization of beta cells) were incubated in both 3.3 mM and 16.7 mM glucose. To investigate the effect of C1 on L-type Ca^{2+} channels, W and GK rat islets were incubated with L-type Ca^{2+} channel inhibitor, 10 µM nifedipine (Sigma-Aldrich, USA). To investigate the effect of protein kinase C (PKC) and protein kinase A (PKA) on C1-induced insulin release, W and GK rat islets were incubated with PKA-inhibitor, H89 (10 uM) (Sigma-Aldrich, USA) or the PKC inhibitor, calphostin-C (1.5 uM) (Sigma-Aldrich, USA) for 60 min in KRB containing 3.3 mM and 16.7 mM glucose.

To assess the possible involvement of exocytotic G-protein in C1-induced insulin release, W and GK rat islets were pretreated at 37°C overnight with 100 ng/ml pertussis toxin, RPMI 1640 culture medium (SVA, Sweden) containing 11 mM glucose (Sigma-Aldrich, USA), 30 mg *L*-glutamine (Sigma-Aldrich, USA), 10% heat-activated fetal calf serum, antibiotics (100 IU/ml penicillin and 0.1 mg/ml streptomycin) (Invitrogen, USA) [14]. The islets which have been exposed overnight with or without pertussis toxin were incubated with C1 at 3.3 mM and 16.7 mM glucose. Aliquots of the medium were analyzed using RIA for the insulin content measurement [16].

2.4 Statistical Evaluations

All values are presented in terms of the mean ± SEM. Difference between experimental groups was analyzed using paired *t*-test. *P*-value of less than 0.05 was

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considered to be significant. All data were analyzed using Prism Graph Pad Software (CA, USA).

3. RESULTS

3.1 Effects of C1 on insulin secretion in rat islets with K-ATP channel opened by diazoxide and depolarized by KCl

In W rat islets, C1 significantly increased insulin release 8.8-fold (P<0.001) and 4.6-fold (P<0.01) compared to the control group at 3.3 mM and 16.7 mM glucose respectively (Table 1). In GK rat islets, C1 significantly stimulated insulin release by 8.4 –fold and 4.3-fold (both P<0.01) at 3.3 mM and 16.7 mM glucose respectively.

The opening of K-ATP channels by adding diazoxide (0.25 mM) in the rat islets inhibited insulin release at 16.7 mM glucose but not at 3.3 mM; W (P<0.01) and GK (P<0.05) compared to control (Table 1). At 16.7 mM glucose, diazoxide decreased insulin response to C1 from 140.8 ± 5.1 to 85.4 ± 5.3 (µU/islet/hour) in W and from 56.2 ± 2.7 to 37.4 ± 3.4 (µU/islet/hour) in GK (both at P<0.01). The addition of KCl in the islets incubated with diazoxide to depolarize the cells significantly increased insulin release in W and GK at 3.3 mM and 16.7 mM glucose.

At 3.3 mM glucose, the insulin secretion of W rat islets incubated with C1+ diazoxide + KCl increased 1.7-fold both compared with C1 (P<0.01) and with diazoxide + KCl (P<0.05). At 16.7 mM, there were significant differences observed in islets incubated with C1+ diazoxide and KCl compared to islets incubated in C1 and islets incubated with diazoxide + KCl (both at P<0.05).

At 3.3 mM glucose, GK rat islets incubated with C1 + diazoxide + KCl significantly increased the insulin secretion 1.5-fold both compared with islets incubated with C1 (P<0.01) and with diazoxide + KCl (P<0.05). At 16.7 mM glucose, the insulin secretion increased by 1.5-fold in islets incubated with C1+ diazoxide + KCl compared to islets incubated in C1 and 1.6-fold when compared with islets incubated with diazoxide + KCl (both at P<0.05).

Table 1: Effect of diazoxide on the insulin response to C1 in the isolated W and GK rat islets. Results of insulin release (µU/islet/h) are the mean ± S.E.M of three batch incubations at each condition.

	W rat islets		**GK rat islets**	
Glucose (mM)	**3.3 mM**	**16.7 mM**	**3.3 mM**	**16.7 mM**
Addition to the medium				
None	2.5 ± 0.3	30.9 ±1.4	1.2 ± 0.1	13 ± 0.2
T.crispa C1 (10 µg/ml)	22.1 ± 0.4***	140.8 ±5.1**	10 ± 0.3**	56.2 ± 2.7**
Diazoxide (0.25 mM)	2.6 ± 0.1	3.6 ± 0.2**	1.2 ± 0.2	10.1 ± 0.5*
Diazoxide (0.25 mM)+KCl (50 mM)	22.8 ± 2.3**	131.2 ± 7.7*	10.2 ± 1**	54 ± 5.5**
T.crispa C1 (10 µg/ml)+Diazoxide (0.25 mM)	23.6 ± 0.7##	85.4 ± 5.3##	9.9 ± 0.6	37.4 ± 3.4##
T.crispa C1 (10 µg/ml) + KCl (50 mM) + Diazoxide (0.25 mM)	38.5 ± 1.2##†	155.7 ± 7.4#†	14.8 ± 0.3##†	84.5 ± 5.8#†

**P<0.05; **P<0.01; ***P<0.001 when compared with control group with no addition*
#P<0.05; ##P<0.01 when compared with group with only T.crispa C1
†P<0.05 when compared with diazoxide+KCl

3.2 Effects of Nifedipine on C1-induced Insulin Secretion in Rat Islets

In W islets at 3.3 mM and 16.7 mM glucose, C1-induced insulin release was 24 ± 3.3 (P<0.05) and 157.5 ± 7.7 (µU/islet/hour)(P<0.01) respectively compared to the control (Table 2). In the GK islets, C1 stimulated insulin release was 10.5 ± 0.6 (P<0.01) and 59.3 ± 5.3 (P<0.05), respectively. Nifedipine (10 µM), a blocker of L-type Ca^{2+} channels, inhibited insulin release in W and GK rat islets at 16.7 mM glucose (Table 2). The insulin release was decreased by nifedipine in W from 30.3 ± 3.1 to 5.3 ± 0.7 (µU/islet/hour) (P<0.05) and from 13.4 ± 0.6 to 4.4 ± 0.1 (µU/islet/hour) (P<0.01) in GK rat islets. In the presence of nifedipine, C1-induced insulin secretion of islets was decreased in W from 157.5 ± 7.7 to 132 ± 10 (µU/islet/hour) (P<0.01) and from 59.3 ± 5.3 to 30.6 ± 3.2 (µU/islet/hour) (P<0.05) in GK rat islets at 16.7 mM glucose.

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Table 2: Effect of nifedipine on the insulin response to C1 in the isolated W and GK rat islets. Results of insulin release (µU/islet/h) are the mean ± S.E.M of three batch incubations at each condition

	W rat islets		GK rat islets	
Glucose	**3.3 mM**	**16.7 mM**	**3.3 mM**	**16.7 mM**
Addition to the medium				
None	2.6 ± 0.2	30.3 ± 3.1	1.3 ± 0.2	13.4 ± 0.6
T.crispa C1 (10 µg/ml)	24 ± 3.3*	157.5 ± 7.7**	10.5 ± 0.6**	59.3 ± 5.3*
Nifedipine (10 µM)	2.3 ± 0.2	5.3 ± 0.7*	1 ± 0.1	4.4 ± 0.1**
T.crispa C1 (10 µg/ml) + Nifedipine (10 µM)	22.2 ± 1.6**	132 ± 10**##	10.3 ± 0.5**	30.6 ± 3.2*#

**P<0.05;* ***P<0.01 when compared with control group with no addition*
#P<0.05; ##P<0.01when compared with group with only C1

3.3 The Effects of H89 and Calphostin C on C1-induced Insulin Secretion in Rat Islets

Incubation of W islets at 3.3 mM and 16.7 mM with C1 stimulated insulin release at 21.4 ± 3.2 (P<0.05) and 152.4 ± 8.3(P<0.01)(µU/islet/hour) respectively (Table 3). In the GK islets, C1 stimulated insulin release at 11.2 ± 1.2 (P<0.05) and 56 ± 2.4 (P<0.01) respectively. No significant difference was observed when W and GK islets were incubated in H89 (10 µM) and calphostin C (10 µM) at both 3.3 mM and 16.7 mM compared to the control group with no addition. H89 and calphostin C inhibitors did not affect the insulin response to C1 respectively in W and GK islets at both 3.3 mM and 16.7 mM glucose.

Table 3:Effect of H89 and calphostin C on the insulin response to C1 in the isolated Wistar and GK rat islets. Results of insulin release (µU/islet per h) are the mean ± S.E.M of three batch incubations at each condition

	W rat islets		Gk rat islets	
Glucose	**3.3 mM**	**16.7 mM**	**3.3 mM**	**16.7 mM**
Addition to the medium				
None	3.1 ± 0.2	31± 3.6	2.4 ± 0.7	15.3 ± 1.2
H89 (10 µM)	4.4 ± 0.3	25.6 ± 1.2	2.1 ± 0.2	15.6 ± 0.9
Calphostin C (1.5 µM)	5.0 ± 0.3	24.1 ± 1.5	1.8 ± 0.4	14.3 ± 1.1
T.crispa C1 (10 µg/ml)	21.4 ± 3.2*	152.4 ± 8.3**	11.2 ± 1.2*	56 ± 2.4**
T.crispa C1 (10 µg/ml) + H89 (10 µM)	20.1 ± 1.8*	159.7 ± 4.9**	8.0 ± 1.0**	45.7 ± 2.8*
T.crispa C1 (10 µg/ml) + Calphostin C (1.5 µM)	21 ± 0.6**	150.5 ± 6.7**	6.6 ± 1.0*	53 ± 2.8*

**P<0.05;* ***P<0.01 when compared with control group with no addition*

3.4 Effect of Pertussis Toxin on Insulin Releasing Effect of C1 in Rat Islets

Pertussis toxin (100 ng/ml) decreased the insulin response to C1 in the W (Figure 2) and GK (Figure 3) rat islets at 16.7 mM glucose only when compared to control group without exposure to pertussis toxin. At 16.7 mM glucose, pertussis toxin decreased the insulin response to C1 in the W islets from 137.1 ± 4.3 to 98.6 ± 5.3 µU/islet/hour (P<0.01) and GK rat islets from 60.7 ± 2.5 to 43.8 ± 0.7 µU/islet/hour (P<0.05).

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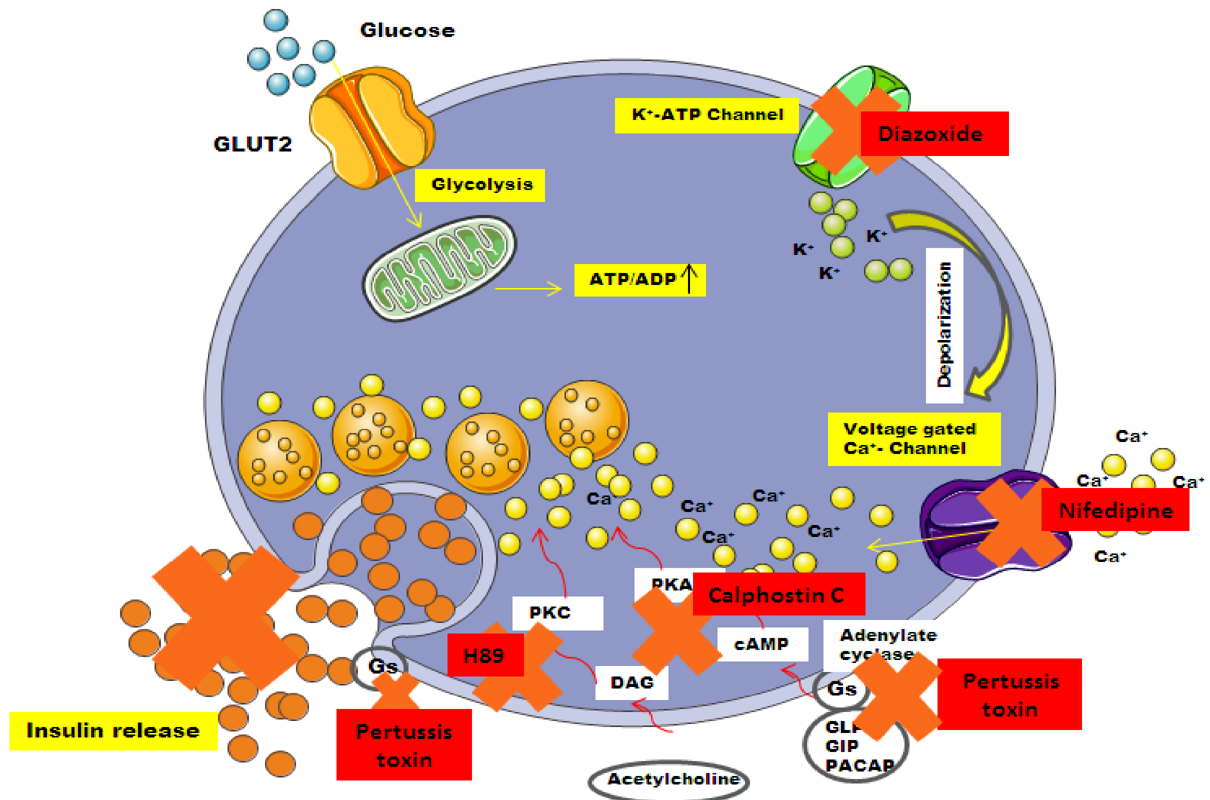

Figure 1: Schematic model of the glucose induced insulin secretion in the pancreatic β-cells. In this study, several inhibitors were used to block the insulin release in order to understand the mechanisms involved by C1. W and GK rat islets were incubated with diazoxide (to open the K-ATP channels), nifedipine (to block the L-type Ca^{2+} channel), H89 and calphostin-C (to block protein kinase A and protein kinase C pathways respectively). Pertussis toxin was used to inhibit insulin release via the Gs-proteins which act on the adenalyte cyclase and on the exocytosis of insulin release.

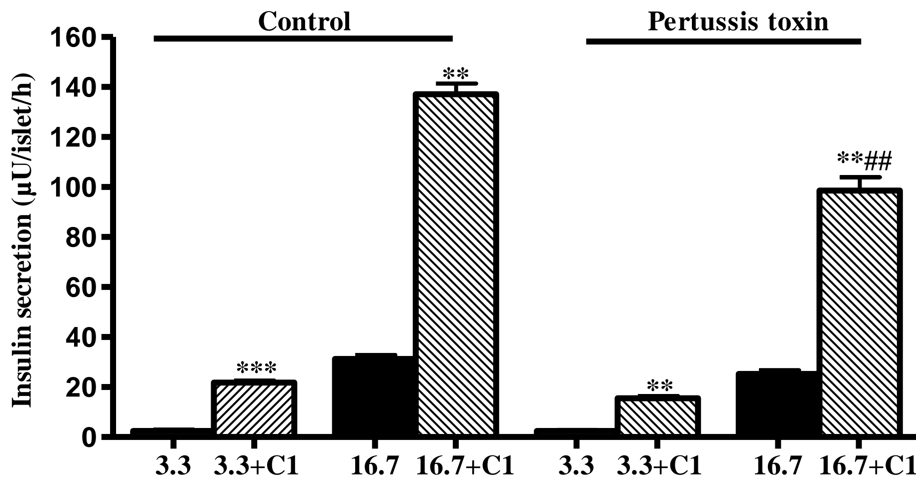

Figure 2: Effect of pertussis toxin on the insulin response to C1 in the isolated W rat islets. Results of insulin release (µU/islet/h) are the mean ± S.E.M of three batch incubations at each condition.

$^{**}P<0.01$; $^{***}P<0.001$; when compared with control group with no addition at 3.3 mM or 16.7 mM glucose

$^{\#\#}P<0.01$ when compared with islets incubated with C1 at 16.7 mM glucose without exposure to pertussis toxin

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Figure 3

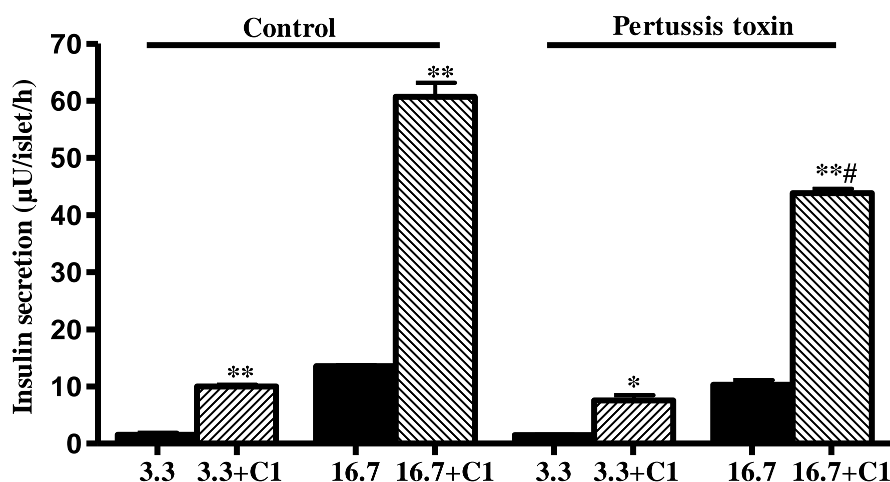

Figure 3: Effect of pertussis toxin on the insulin response to C1 in the isolated GK rat islets. Results of insulin release (μU/islet/h) are the mean ± S.E.M of three batch incubations at each condition.
$^{*}P<0.05$; $^{**}P<0.01$ when compared with control group with no addition at 3.3 mM or 16.7 mM glucose
$^{\#}P<0.05$ when compared with islets incubated with C1 at 16.7 mM glucose without exposure to pertussis toxin

4. DISCUSSION

When exploring the mechanisms of insulin release in W and GK rat islets, we showed that C1 significantly stimulated insulin release at both low and high glucose. The mechanism by which C1 stimulates insulin release in W and GK was partly mediated via K-ATP and L-type Ca^{2+} channels rat islets. The effect of C1 was also partly dependent on pertussis toxin sensitive G_e-protein at low and high glucose. C1 effect was not mediated via the PKA and PKC system.

We recently demonstrated that an oral administration of C1 decreased the blood glucose and increased serum insulin levels in W and GK rats. We showed that the blood glucose lowering effect was associated with the capability of C1 to stimulate insulin secretion in isolated W and GK rats in a dose- and glucose-dependent manner [13]. Therefore, to understand the mechanism of action of C1 on the pancreatic of W and GK pancreatic β-islets, we explored the insulin secretion via K-ATP dependent pathway.

In the K-ATP dependent pathway, the elevated glucose metabolism increases the cellular adenosine triphosphate/adenosine diphosphate (ATP-ADP) ratio which results in the closure of ATP-sensitive K^+ (K-ATP) channels. This will lead to beta cell membrane depolarization which results in the opening of the voltage-dependent Ca^{2+} channels, increase in the Ca^{2+} influx and triggers insulin release [17-19].

W and GK islets were incubated with diazoxide, which acts on the K-ATP site by opening of the K-ATP channels, and thus inhibits insulin release [5]. Our results showed that the incubation of islets with diazoxide significantly decreased insulin release in W and GK rat islets at high (*P*<0.05) glucose only. Diazoxide significantly decreased the insulin response to C1 at high glucose in both W and GK rat islets compared with islets incubated with C1, indicating that C1 might act partly on the K-ATP channel. The addition of KCl into islets incubated with diazoxide to depolarize the membrane significantly increased insulin release in W and GK rat islets at low and high glucose due to the Ca^{2+} entry via voltage-sensitive Ca^{2+} channels which promotes insulin release. KCl initiates membrane depolarization [5], which leads to the increase in Ca^{2+} concentration followed by stimulation of insulin secretion [20]. Furthermore, in both W and GK rat islets, the presence of KCl significantly increased the insulin response to C1 at high and low glucose. Our result showed that the effect of C1 is partly in contrast to the sulfonylureas drug which stimulates insulin by closing the K-ATP channel [21, 22].

In further experiments, islets were incubated with nifedipine which is closing the Ca^{2+} channels and thus block the influx of Ca^{2+} from entering the cells and thereby inhibit insulin release [23]. Our results showed that in the presence of nifedipine, the insulin release was significantly decreased in W and GK rat islets at high glucose only. At 16.7 mM glucose, nifedipine decreased the insulin response to C1 in W and GK rat islets compared with islets incubated with C1 only suggesting that the effect of C1 might partly involve the L-type Ca^{2+} channels. A previous study showed that the insulin release of *T.crispa* was inhibited by the channel blockers, verapamil and nifedipine suggesting that the effect of *T.crispa* extract was mediated by modulation of Ca^{2+} handling in the beta cells [24].

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While an increase in intracellular Ca^{2+} concentration is the principal signal, other intracellular signals such as cAMP and DAG also are important to regulate and modulate exocytosis through protein phosphorylation mediated by PKA and PKC respectively [9,18]. In this study, the PKA inhibitor, H89 and PKC inhibitor, calphostin C, did not affect the insulin secretion at both high and glucose in W and GK rat islets when compared to the control group. When C1 was added to H89 and calphostin C respectively, the insulin-stimulating effect was not blocked indicating that the effect of C1 did not involve PKA and PKC systems. Glucose may activate PKA through triggering of the K^+-ATP channel-dependent pathway. Glucose amplification of K^+-ATP channel-independent insulin secretion, on the other hand, occurs by PKA-independent mechanisms.

G-proteins are involved in signal transduction in many tissues and act as mediators of hormonal inhibition of insulin release [25,26]. G_i protein which is known to be involved in the inhibition of adenylyl cyclase is inhibited by pertussis toxin (expressed by *Bordetella pertussis*) via ADP-ribosylation [26,27]. G_e protein acts on the exocytosis of insulin release in the pancreatic islets [11]. When compared to control group, the pre-incubation of islets with pertussis toxin decreased the insulin response to C1 in W and GK rat islets at 16.7 mM glucose. This suggests that C1 acts on pertussis toxin sensitive G_e-proteins.

5. CONCLUSIONS

When exploring the mechanisms of insulin release in the W and GK pancreatic islets, we showed that C1 had an effect partly via K-ATP channels since diazoxide partly, but not totally suppressed C1 stimulation at 16.7 mM glucose. C1 effect was also dependent on L-type Ca^{2+} channels since nifedipine suppressed the insulin response to C1 at 16.7 mM. C1 effect was partly dependent on pertussis toxin sensitive G_e-protein. There was no modulation by PKA and PKC inhibitors since H89 and calphostin C did not affect the insulin response to C1. The major stimulatory effect of C1 might be on the exocytosis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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