A novel peptide Phylloseptin-PBu from Phyllomedusa burmeisteri possesses insulinotropic activity via potassium channel and GLP-1 receptor signalling

A novel peptide Phylloseptin-PBu from *Phyllomedusa burmeisteri* possesses insulinotropic activity via potassium channel and GLP-1 receptor signalling

Qilin Long | Lei Wang | Mei Zhou | Yuxin Wu | Tianbao Chen

Abstract
Insulin, as one of the most important hormones regulating energy metabolism, plays an essential role in maintaining glucose and lipid homeostasis *in vivo*. Failure or insufficiency of insulin secretion from pancreatic beta-cells increases glucose and free fatty acid level in circulation and subsequently contributes to the emergence of hyperglycaemia and dyslipidaemia. Therefore, stimulating the insulin release benefits the treatment of type 2 diabetes and obesity significantly. Frog skin peptides have been extensively studied for their biological functions, among which, Phylloseptin peptides discovered in Phyllomedusinae frogs have been found to exert antimicrobial, antiproliferative and insulinotropic activities, while the mechanism associated with Phylloseptin-induced insulin secretion remains elusive. In this study, we reported a novel peptide named Phylloseptin-PBu, isolated and identified from *Phyllomedusa burmeisteri*, exhibited dose-dependent insulinotropic property in rat pancreatic beta BRIN-BD11 cells without altering cell membrane integrity. Further mechanism investigations revealed that Phylloseptin-PBu-induced insulin output is predominantly modulated by K<sub>ATP</sub>-[K+] channel depolarization triggered extracellular calcium influx and GLP-1 receptor initiated PKA signalling activation. Overall, our study highlighted that this novel Phylloseptin-PBu peptide has clear potential to be developed as a potent antidiabetic agent with established function-traced mechanism and low risk of cytotoxicity.

KEYWORDS
calcium, GLP-1 receptor, insulin release, peptide, PKA signalling, potassium channel

1 INTRODUCTION

Type 2 diabetes mellitus (T2DM), accounting for almost 90% cases of the disease, has become a global public health threat for both individuals and social healthcare systems. Although the aetiology of T2DM has not been fully understood, it is widely accepted that deficiency of insulin secretion from pancreatic beta-cells accompanied by attenuation/abnormality of insulin sensitivity in specific tissues (liver, heart, fat, skeleton muscle) is the common feature of T2DM. Either beta-cell disorder or decreased beta-cell mass contributes to the impairment of insulin release. Hyperglycaemia and dyslipidaemia, as hallmarks of T2DM, appear when the blood glucose and lipid lowering effect of secreted insulin is not sufficient to compensate for insulin resistance. When patients suffer the long-term pathological conditions, complications of T2DM, such as heart and blood vessel diseases, neuropathy, nephropathy, eye/foot/hearing/skin damage and Alzheimer’s disease, will occur.
Clinically, the therapies for the treatment of T2DM are aimed at decreasing hepatic glucose production, improving insulin resistance and increasing insulin release. In particular, for the insulin secretion boosting therapy, two types of drugs are currently prescribed, one acts on closing the K_ATP channels on the beta-cells for the calcium influx, and the other type targets on mimicking or enhancing the effect of gut hormones (also named as incretins),\(^7\) a good example of the latter type is extendin-4, it was firstly isolated and characterized from the Heloderma suspectum venom, the glycaemic-control action of extendin-4 is via the similar way with glucagon-like peptide 1 (GLP-1), which mainly attributes to the potentiation of insulin secretion in a glucose-sensing manner.\(^7\)-\(^14\)

Bioactive peptides from amphibian secretions and venoms with characteristics of diversity and specificity provide candidates for the development of novel drugs for combating multiple diseases, including antibiotic-resistant pathogens caused infections, cancers and T2DM.\(^15\) Previous elegant research has reported that some frog skin-derived peptides possess the ability to stimulate insulin release both in vitro and in vivo at relatively low concentration and dose without causing cellular toxicity.\(^16\)-\(^18\) Many have been extensively studied in the fields of amino acid isoform replacement, post-translational modification and primary structure modification, all of these focus on increasing the insulin release efficiency, preventing the degradations caused by endogenous enzymes and improving the cytotoxicity.\(^16\),\(^19\),\(^20\) Nevertheless, the mechanism associated with how these bioactive peptides are involved in the insulin release action has rarely been investigated. Thus, in this study, we report a novel insulinotropic peptide isolated from Phyllomedusa burmeisteri, in which species such peptide has never been identified, belongs to Phylloseptin family, named as Phylloseptin-PBns. At low concentration, it showed glucose-comparably insulin secretion stimulatory effects (5 \(\mu\)M, 4.5 \(\times\) 250 mm, Phenomenex, Macclesfield, Cheshire, UK) using a gradient program. The gradient elution formed from TFA/water (0.05/99.95, v/v) to TFA/water/1% Acetonitrile (0.05/19.95/80.00, v/v/v) in 240 minutes at a flow rate of 1 mL/min, and the effluent was detected by UV absorbance at 214 and 280 nm. Fractions were collected at 1-minutes interval by an automatic fraction collector (GE Healthcare, Little Chalfont, UK). The molecular mass of each fraction was analysed using the matrix-assisted laser desorption ionization time of flying (MALDI-TOF) mass spectrometer (Voyager DE, PerSeptive Biosystems, Foster City, CA, USA) with \(\alpha\)-cyano-4-hydroxycinnamic acid (CHCA) matrix in positive detection mode. Fractions with the molecular weight coincidence with the mature peptide which is predicted from cloned cDNA were then subjected to primary structural analysis by MS/MS fragmentation using a liquid chromatography quadrupole (LCQ)-Fleet electrospray ion-trap mass spectrometer (Thermo Fisher Scientific, San Francisco, CA, USA).

### 2.2 "Shotgun" cloning of a novel Phylloseptin-like peptide from Phyllomedusa burmeisteri skin secretion

Five milligrams of lyophilized *Phyllomedusa burmeisteri* secretion powder was dissolved in 1 mL cell lysis/binding buffer to isolate polyadenylated mRNA by magnetic oligo-dT beads via using Dynabeads\(^{20}\) mRNA DIRECT\(^\text{TM}\) Kit (Dynal Biotech, Liverpool, UK). The reverse-transcription products were subjected to 3'-RACE PCR procedures to acquire the full length of preproprotein nucleic acid sequences using a SMART-RACE kit (Clontech, Palo alto, CA, USA). Briefly, a nested universal primer (NUP) (supplied with the kit) and a degenerate primer (S1: 5'-ACTTTCYGAWTRYAAGMCCAAABAT G-3', Y = C + T, W = A + T, R = A + G, M = A + C, B = T + C + G) which was designed on the basis of the 5'-untranslated region of phylloxin cDNA from *Phyllomedusa bicolor*.\(^22\) The PCR cycling was carried out as follows: initial denaturation step: 90 seconds at 94°C; 35 cycles: denaturation 30 seconds at 94°C, primer annealing for 30 seconds at 58°C; extension for 180 seconds at 72°C. PCR products were gel-purified and cloned using a pGEM-T Easy vector system (Promega Corporation, Southampton, UK), and then, selected samples were sequenced using an ABI 3100 automated sequencer (Applied Biosystems, Foster City, CA, USA).

### 2.3 Identification and structural characterization of the novel Phylloseptin-like peptide from Phyllomedusa burmeisteri skin secretion

An aliquot sample of lyophilized skin secretion was dissolved in 1 mL trifluoroacetic acid (TFA)/water (0.05/99.95, v/v) and clarified by centrifuge. One millilitre supernatant was decanted and pumped into reversed phase-HPLC column (Phenomenex C-5, 300 Å, 5 \(\mu\)m, 4.5 \(\times\) 250 mm, Phenomenex, Macclesfield, Cheshire, UK) using a gradient program. The gradient elution formed from TFA/water (0.05/99.95, v/v) to TFA/water/Acetonitrile (0.05/19.95/80.00, v/v/v) in 240 minutes at a flow rate of 1 mL/min, and the effluent was detected by UV absorbance at 214 and 280 nm. Fractions were collected at 1-minutes interval by an automatic fraction collector (GE Healthcare, Little Chalfont, UK). The molecular mass of each fraction was analysed using the matrix-assisted laser desorption ionization time of flying (MALDI-TOF) mass spectrometer (Voyager DE, PerSeptive Biosystems, Foster City, CA, USA) with \(\alpha\)-cyano-4-hydroxycinnamic acid (CHCA) matrix in positive detection mode. Fractions with the molecular weight coincidence with the mature peptide which is predicted from cloned cDNA were then subjected to primary structural analysis by MS/MS fragmentation using a liquid chromatography quadrupole (LCQ)-Fleet electrospray ion-trap mass spectrometer (Thermo Fisher Scientific, San Francisco, CA, USA).

### 2.4 Solid phase peptide synthesis of Phylloseptin-PBns

Once the primary structural of the Phylloseptin-PBns was unequivocally confirmed through both MS/MS fragmentation sequencing and
molecular cloning, the solid-phase Fmoc strategy was adopted via Tribute™ automated solid-phase peptide synthesizer 4 (Protein Technologies, Tucson, AZ, USA) to synthesize the novel peptide as previously described.23 The synthesized peptide replicates were purified by reverse phase HPLC with C18 column (Jupiter C18, 5 μm particle, 300 A pore, 250 × 10 mm, Phenomenex, UK), and both molecular masses and high-purity of the synthetic replicates were confirmed via MALDI-TOF.

### 2.5 Determination of peptide secondary structures using circular dichroism (CD) analysis

CD measurements were performed using a JASCO J-815 CD spectrometer (Jasco, Essex, UK) at room temperature (20-25°C). Peptide was dissolved in (i) water, (ii) 50% (v/v) trifluoroethanol (TFE)-water and (iii) 50% (v/v) Methanol-water to reach a final concentration of 100 μmol/L before transferred and measured in a 0.1-cm-high precision quartz cell (Hellma Analytics, Essex, UK). The wavelength was confirmed via MALDI-TOF. The corresponding wavelength (nm), which is calculated from the measured bandwidth and data pitch are 1 and 0.5 nm, respectively. CD data were collected from acute insulin release assay.

### 2.6 Cell line and cell culture

The BRIN-BD11 rat clonal beta-cell line is purchased from European Collection of Authenticated Cell Cultures (Catalogue No. 10033003, UK), which serves as a canonical in vitro model to study insulin release in response to nutrients and chemicals.27 After resuscitation, BRIN-BD11 cells were cultured using RPMI-1640 medium (Invitrogen, Paisley, UK) containing 10% (v/v) foetal bovine serum (FBS) (Gibco®, UK), 10 mmol/L N2-sodium pyruvate, 100 mmol/L NaHCO3, 100 mmol/L NaF, 10 mM Na2P2O7, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin and 10 μg/mL leupeptin. After centrifugation at 14 000 × g for 10 minutes at 4°C, the supernatant was boiled for 5 minutes in SDS-PAGE sample buffer [50 mmol/L Tris·Cl (pH 6.8), 2% sodium dodecyl sulphate, 10% glycerol, 2% β-mercaptoethanol and 0.004% bromophenol blue] and separated by 7.5% SDS-PAGE, and transferred to nitrocellulose membrane (GE Healthcare Life sciences, UK), and detected by Western blotting with the indicated antibody (Cell Signaling Technology, UK) using enhanced chemiluminescence [Pierce® ECL Western Blotting Substrate (Thermo scientific, USA)], and imaged via Chemi Doc™ MP system (Bio-Rad, UK). The antibodies that chosen for the present study were PKA-Cα (1:1000; Cell Signaling, #4782), phospho-PKA-C (Thr197) (1:1000; Cell Signaling, #5661), GAPDH (1:1000; Cell Signaling, #5174) and Anti-rabbit IgG, HRP-linked Antibody (1:2000; Cell Signaling, #7074).
2.10 | Statistical analysis

All results are presented as mean ± SEM determined by two-tailed Student’s t test or one-way ANOVA. Pairs comparisons of the means were made, and P < .05 was regarded as a significant difference. The Bonferroni method was used to adjust the observed significance levels for the fact the multiple contrasts were being tested.

3 | RESULTS

3.1 | Primary structure identification and synthesis of novel peptide Phylloseptin-PBu

From the skin secretion of Phyllomedusa burmeisteri-derived cDNA library, the cDNA encoding the biosynthetic precursor of Phylloseptin-PBu consistently and repeatedly cloned. The coding region of the cDNA consisted of 66 amino acids which can be divided into four domains: the putative signal peptide, which is located at the 5’-terminus with 22 amino acids; the 24-residue acidic spacer domain, a typical Lys-Arg (K-R) propeptide convertase processing site and the C-terminus, was successfully detected by LCQ MS/MS. Taken ended with amidation, a post-translational modification at the peptide. Although the insulinotropic effect of Phylloseptin-PBu is weaker compared to L-alanine, which mainly induces insulin output via Ca2+ influx mechanism,29 it still prompted us to explore the signal pathways that involved in this novel Phylloseptin-PBu peptide exhibited bioactivity.

As Phylloseptin-like peptides have been reported to exert antimicrobial activities against Gram-positive and Gram-negative bacterial...
We therefore investigated whether Phylloseptin-PBu-triggered insulin release was caused by the damage to cell membrane. The minimal inhibitory concentration (MIC) assay implied that Phylloseptin-PBu possessed no antimicrobial activity against *S. aureus*, *E. coli* and *C. albicans* when the concentration was up to 10⁻⁷ mol/L (Figure S2). The lactate dehydrogenase (LDH) tests suggested that Phylloseptin-PBu treatment did not compromise cell membrane integrity in BRIN-BD11 cells at concentrations up to 10⁻⁷ mol/L (Figure 3B). Further haemolytic measurement using horse blood cell also did not show cytolytic effect of Phylloseptin-PBu at 10⁻⁷ mol/L (Figure 3C). Therefore, these results lead us to investigate the specific mechanism that related to insulin release effect of Phylloseptin-PBu.

### 3.4 Potassium and calcium channel-modulated Ca²⁺ influx examination

As indicated, elevated intracellular ATP level and increased ATP/ADP ratio leads to the closure of potassium channel, induction of cell

---

**TABLE 1** Helicity index of Phylloseptin-PBu analysed according to CD spectra data using DICHROWEB online server

<table>
<thead>
<tr>
<th>Percentage of helicity (%)</th>
<th>α-helix</th>
<th>β-sheet</th>
<th>Random coil</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>7</td>
<td>51</td>
<td>42</td>
</tr>
<tr>
<td>50% TFE + H₂O</td>
<td>96</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>50% Methanol + H₂O</td>
<td>8</td>
<td>45</td>
<td>47</td>
</tr>
</tbody>
</table>
membrane depolarization and opening of voltage-gated calcium channel. These actions promote extracellular Ca\(^{2+}\) influx, which plays a key role in mediating L-alanine-activated insulin release from BRIN-BD11 cells.\(^{31}\) We employed diazoxide (activator of ATP-sensitive potassium channels), verapamil (inhibitor of voltage-gated calcium channels) and Ca\(^{2+}\)-free medium to examine whether Phylloseptin-PBu stimulated insulin release is via Ca\(^{2+}\) mobilization. As shown in Figure 4, pre-administration of diazoxide and verapamil significantly attenuated Phylloseptin-PBu-induced insulin production, deprivation of Ca\(^{2+}\) significantly attenuated the insulin producing activity in all tested groups, but significant enhancement of insulin release was still observed after Phylloseptin-PBu treatment even culturing the cells with Ca\(^{2+}\)-free KRB buffer with or without diazoxide or verapamil. Together, these results suggested that calcium influx, mediated by ATP-sensitive potassium channel induced depolarization and calcium channel opening, played the dominant role in the insulinotropic activity of Phylloseptin-PBu, nevertheless, this effect was not completely abrogated, it prompted us to hypothesize that other signalling might be involved.

3.5 PKA signalling activation-modulated insulin secretion examination

Activation of cAMP/PKA was also reported to elicit insulin secretion in islet.\(^{32}\) Thus, we interrogated the PKA signalling using the PKA

![Figure 4](image-url)
activator (IBMX, 3-Isobutyl-1-methylxanthine) and inhibitor (H89) in the presence or absence of Phylloseptin-PBu. The results (Figure 5A) showed that although H89 significantly blunted Phylloseptin-PBu-induced insulin secretion, compared with singular H89 treatment, Phylloseptin-PBu still has a trend to increase insulin release. While IBMX alone and co-administered with Phylloseptin-PBu increased and dramatically further enhanced insulin output, respectively. Based on these observations, we speculated that PKA signalling is not only modulated by Phylloseptin-PBu, but also L-alanine for the stimulation of insulin release from BRIN-BD11 cells.

Furthermore, we used Western blotting to examine the effect of Phylloseptin-PBu vs IBMX on PKA activation in BRIN-BD11 cells. As shown in Figure 5B, the phosphorylation level of PKA at Thr197 was markedly elevated by IBMX and Phylloseptin-PBu, which confirmed the involvement of PKA cascade in Phylloseptin-PBu-induced insulinotropic activity.

**FIGURE 5**  A, Acute effects (20 min stimulation) of $10^{-7}$ mol/L Phylloseptin-PBu on the insulin release. BRIN-BD11 cells were incubated with 1.1 mmol/L glucose, 5.6 mmol/L glucose, 16.7 mmol/L glucose, 10 mmol/L alanine, 10 µmol/L H89, 200 µmol/L IBMX and $10^{-7}$ mol/L Phylloseptin-PBu in Ca²⁺-contained KRB buffer. Data were analysed with unpaired Student’s t test using GraphPad Prism 5 software. Values are the mean ± SEM for three independent experiments. **P < .01, ***P < .001 between indicated groups. B, BRIN-BD11 cells were treated with 100, 200 µmol/L IBMX or $10^{-7}$ mol/L Phylloseptin-PBu for 2 h, total proteins were extracted. Eighty µg protein was immunoblotted to determine the abundance of total PKA and phosphor-PKA at Thr 197. Signal intensity was quantified by Image Lab software for statistical comparison, *P < .05, **P < .01 vs vehicle control.
3.6 | Assessment of GLP-1 receptor activation by Phylloseptin-PBu

Next, we investigated how Phylloseptin-PBu activated PKA signalling. As GLP-1 and extendin-4 have been demonstrated to enhance insulin release in a PKA-dependent manner,7,33-35 we hypothesized that Phylloseptin-PBu act as an agonist to GLP-1 receptor and contributes to cAMP/PKA signalling activation. Following on from this, we used GLP-1 receptor antagonist exendin-(9-39) to examine whether it can impair Phylloseptin-PBu’s action. As the results shown in Figure 6, the insulin secretion effect was significantly decreased in both exendin-(9-39) and exendin-(9-39) with Phylloseptin-PBu co-incubation groups; nonetheless, the latter group also showed a prominent higher insulin output. While further consistently activating KATP channels with diazoxide or blocking the calcium channels with verapamil showed an additive insulin secretion inhibition effect. In the meantime, the calcium deprivation patterns, again, exhibited significant lower insulin production, of note is that under such conditions, Phylloseptin-PBu still stimulated higher insulin production. As a whole, these results confirmed that Phylloseptin-PBu could interact with GLP-1 receptor as a partial agonist to active PKA signalling for modulating Ca2+ channels, promoting insulin secretion. However, a Ca2+ and PKA signalling-independent mechanism induced by Phylloseptin-PBu was likely to play a role in this action.

3.7 | Other mechanism examination for the induction of insulin release by Phylloseptin-PBu

In order to assess whether other signalling is existed in regulating Phylloseptin-PBu’s insulotropic activity, we treated the cells using diazoxide or verapamil together with H89, and measured the insulin release. As the Figure 7 shown, compared with singular H89 treatment, Phylloseptin-PBu still has moderately, but significantly higher insulin release activity even co-treated with H89 and/or with diazoxide/verapamil, but this effect was completely abolished in the Ca2+-free medium settings, which proposed a KATP- and PKA-independent Ca2+ influx mechanism.

4 | DISCUSSION

In the postprandial state, elevated blood glucose is sensed by pancreatic beta-cells which in response secrete insulin to maintain glucose homeostasis. This process occurs under both physiological and pathological conditions, and thus, increasing insulin release has promising therapeutic potential.5 The mechanism correlated with acute insulin secretion in islets has been well-studied, which is commonly summarized as two distinct pathways, one is related to the KATP channel closure, and
membrane depolarization-triggered intracellular calcium rise via Ca^{2+} channels on both cell membrane and endoplasmic reticulum, the other one is through Ca^{2+}-induced insulin secretory vesicle exocytosis. In this study, we investigated the K_{ATP}-channel, calcium channel, PKA signalling and GLP-1 receptor in regulating Phylloseptin-PBu-induced insulinotropic effect in BRIN-BD11 cells, we confirmed that this novel peptide stimulates acute insulin release at physiological concentration of 5.6 mmol/L glucose by deactivating K_{ATP} channels, leads to the opening of calcium channels and calcium influx, which subsequently drives the fusion of secretory vesicles to the plasma membrane for exocytosis of insulin. We also confirmed that Phylloseptin-PBu partially stimulates GLP-1 receptor, resulting in PKA activation for the increased insulin secretion. Notwithstanding, it must be noticed that, in Figure 5, Ca^{2+}-free medium remarkably decreased insulin output even under the condition of blocking calcium channel using verapamil, suggested a novel cytosol Ca^{2+} import mechanism, which is further supported by the independent results of Figure 7, and we suppose that ionotropic receptors (also known as ligand-gated ion channels) might play the crucial role in it, which warrants further investigations. Moreover, based on Figure 5 and part Figure 6 results, the novel peptide still enhanced insulin release significantly compared with no Phylloseptin-PBu treatment groups, which implied an intracellular Ca^{2+} store (endoplasmic reticulum)-referred mechanism induced by Phylloseptin-PBu. In addition, another interesting issue is that, in Figure 5A, we observed the additive augment insulin release effect of Phylloseptin-PBUs with IBMX, we presume that IBMX, as a phosphodiesterase inhibitor to raise intracellular cAMP, has distinct PKA activation mechanism with Phylloseptin-PBUs, which involves activating of GLP-1 receptor, and therefore, the hyperactive PKA by both Phylloseptin-PBUs and IBMX contributed to the dramatically increased insulin secretion.

In consideration of the secondary structure of Phylloseptin-PBUs, which could also be crucial for its biological relevance, we conducted the CD analysis, the results showed that in 50% TFE aqueous solution, this novel peptide formed a well-defined $\alpha$-helical structure, while in polar solvent, much less of (7%) $\alpha$-helicity was presented. This result was similar to glucose-dependent insulinotropic polypeptide (GIP), GLP-1, extendin-4 and glucagon, likewise, we assume that the full length of $\alpha$-helical conformation might be important for Phylloseptin-PBUs to exert the insulin induction activity. Nonetheless, further NMR or other structure-activity relationship studies are needed for determination.

In addition, we also measured the insulinotropic activity of Phylloseptin-PBUs in 16.7 mmol/L glucose conditions to test whether it could further enhance insulin release, the results showed that no higher insulin release was observed (Figure S3). We speculate that the high glucose level might induce the cells to be stressful, and in such condition, the capacity of insulin release from cells could be limited by the high baseline insulin release.

Overall, in this project, we isolated and identified a novel peptide from Phylomedusa burmeisteri, the insulinotropic effect of this novel peptide Phylloseptin-PBu has been substantially studied, we confirmed the energy-mediated K_{ATP} and Ca^{2+} channels, and the GLP-1 receptor initiated PKA activation involvements in this effect, which has never been published from amphibians sources, our study continuously provide novel candidate and fundamental understanding for the treatment of T2DM in future.

ACKNOWLEDGEMENTS

This work was supported by the internal funding for Natural Drug Discovery Group from School of Pharmacy, Queen’s University Belfast.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTION

Yuxin Wu, Tianbao Chen, Lei Wang and Mei Zhou conceived and designed the experiments. Qilin Long performed the experiments. Qilin Long and Yuxin Wu analysed the data. Tianbao Chen and Lei Wang contributed reagents/materials/analysis tools. Qilin Long and Yuxin Wu wrote the paper. Yuxin Wu and Tianbao Chen edited the manuscript.

ORCID

Yuxin Wu http://orcid.org/0000-0002-2657-8498

REFERENCES


