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IPP-1 controls Akt/CREB phosphorylation extension in A_{2a} adenosine receptor signaling cascade in MIN6 pancreatic β-cell line

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ABSTRACT

Signaling through A_{2a} adenosine receptor specifically prevent pancreatic β-cells (PBCs) loses under diabetogenic conditions. However, signaling mediators of this receptor in PBCs remained unidentified. Thus, we aimed to investigate the possible involvement of PKA/Akt/IPP-1/CREB pathway in MIN6 β-cells. In addition, we investigated IPP-1 role in A_{2a} receptor signaling pathway. The expression of A_{2a} receptor in MIN6 cell line was evaluated by RT-PCR and its functionality confirmed by quantification of cAMP in response to the CGS 21680, an A_{2a} receptor agonist. MTT and Brdu assays were used to evaluate cell viability and proliferation, respectively. PKA activity and insulin release were evaluated using ELISA methods. P-Akt/Akt, p-IPP-1/IPP-1, and p-CREB/CREB levels were assessed using western blotting. IPP-1 knock down assessments was performed using specific siRNA. Our result revealed that MIN6 cells express A_{2a} receptor which actively increased cAMP levels (with EC_{50} = 2.41 μM) and PKA activity. Activation of this receptor increased cell viability, proliferation and insulin release. Moreover, we mentioned A_{2a} receptor stimulation increased p-Akt, p-IPP-1, and p-CREB levels in dose (max at 10 μM of CGS 21680) and time (max at 30 min after CGS 21680 treatment) dependent manner. Interestingly, herein, we found in IPP-1 knocked down cells, A_{2a} receptor failed to activate Akt and CREB. Altogether, we mentioned that in MIN6 cells A_{2a} receptor increase cell viability, proliferation and insulin release through PKA/Akt/IPP-1/CREB signaling pathway. In addition, we conclude A_{2a} receptor signaling through this pathway is dependent to activation of IPP-1.

1. Introduction

Pancreatic β-cells (PBCs) have high adaptability in regeneration and proliferation during pathophysiological situations (Andersson, 2014). This adaptation, at early stages, associated with increase in β-cells mass and insulin secretion (Donath et al., 2005). However, prolonged glucolipotoxicity hampers PBCs proliferation and induce apoptosis (Cnop et al., 2005; Donath et al., 2005). In this regards, finding new signaling targets and small molecules can be helpful for promotion of β-cells proliferation and maintenance (Chen et al., 2009; Gu et al., 2010; Shen et al., 2013; Taylor et al., 2013; Tsuji et al., 2014; El Ouaamari et al., 2016).

Most recently, signaling of Adenosine has been demonstrated to increase β-cells proliferation specifically (Annes et al., 2012; Navarro et al., 2017). Four subtypes of G protein–coupled receptors (GPCRs), A1, A_{2a}, A2b, and A3, engaged in extracellular signaling of this molecule (Capote et al., 2015). Interestingly, diabetic conditions may alters expression and signaling patterns of these receptors in different tissues (Grden et al., 2007; Antonioli et al., 2015). These receptors expressed in isolated PBCs and in many insulinoma cell lines and their activation may influence PBCs viability and insulin secretion (Ohtani et al., 2013). Some of these receptors like A_{2b} and A_3 induce apoptosis in many cells (Jafari et al., 2017, 2018). However, based on whole-organism screening in diabetic zebrafish models, it has been shown that proliferative effects of Adenosine on PBCs possibly mediated through A_{2a} receptor (Andersson et al., 2012). A_{2a} receptor activation by 5′-N-Ethylcarboxamidoadenosine (NECA) agonist, stimulated PBCs proliferation and lowered blood glucose in diabetic mice (Antonioli et al., 2015). The ablation of Adora2a gene decreased baseline level of PBCs proliferation, insulin content and secretion during gestational diabetes (Schulz et al., 2016) and high-fat-diet (HFD)-induced metabolic disorder in mice (Csóka et al., 2017). Interestingly, administration of specific A_{2a} receptor agonist (CGS 21680) improved glucose homeostasis in obese mice (DeOliveira et al., 2017). However, intracellular signaling mediators of this receptor in PBCs are not determined.

Recently, it has been shown that Protein Phosphatase 1 Regulatory Inhibitor Subunit 1 A (PPP1R1A) (Inhibitor-1 or IPP-1) highly expressed in PBCs, nor in α- or exocrine cells of pancreas (Lilja et al.,

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2005; Jiang et al., 2013). Its expression was significantly decreased during chronic hyperglycemia, but acute exposure (24 h) of human islets to high glucose up-regulated its expression. Serum levels of this protein in type 2 diabetic patients had positive and negative correlation with insulin secretion and HbA1c, respectively (Taneera et al., 2014). It is believed that IPP-1, in a complex with protein phosphatase 1 (PP1) and eIF2α, enable sophisticated regulation of insulin biosynthesis (Vander Mierde et al., 2007).

Recent studies showed promising effects of A_{2a} receptor on PBCs proliferation and insulin secretion; however, signaling mediators of this receptor in PBCs remained unidentified. Thus, in this study, we aimed to investigate the possible involvement of PKA/Akt/IPP-1/CREB pathway in A_{2a} receptor mediated signaling in MIN6 β-cells. In addition, role of IPP-1 in A_{2a} receptor signaling pathway was evaluated using siRNA mediated knock down experiments.

2. Material and methods

2.1. Chemical and assay kits

CGS 21680 (Cat: 1063) was purchased from Tocris Bioscience (United Kingdom). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Cat: M2128) and 3,7-dimethyl-1-propargylxanthine (DMPX, an A_{2a} receptor antagonist) (Cat: D134) were purchased from Sigma Aldrich company (Germany). Brdu assay kit (Cat: 6813S) from Cell signaling technology, Mouse Insulin ELISA kit (Cat: 10–1247–01) from Mercodia, PKA Kinase activity assay kit (Cat: ab139435) from Abcam, and Cyclic AMP ELISA Kit (Cat: 581001) from Cayman chemical companies were provided. Fura 2/AM (CAS 108964–32-5) was provided from Santa Cruz (America). Goat anti-IPP1 (sc-15346), goat anti-phospho-IPP-1$^{(Thr35)}$ (sc-14267), mouse anti-CREB-1 (sc-271), goat anti-phospho-CREB$^{(Ser133)}$ (sc-7978), mouse anti-Akt1/2/3 (sc-81434), mouse anti-phospho-Akt1/2/3$^{(Ser473)}$ (sc-81433), mouse anti-Bcl-2 (sc-7382), mouse anti-β-actin (sc-47778), goat anti-mouse IgG-HRP (sc-2031), mouse anti-goat IgG-HRP (sc-2354), and mouse anti-rabbit IgG-HRP (sc-2357) were provided from Santa Cruz Biotechnology. All of siRNA transfection reagents including: IPP-1 siRNA (m) (sc-45874), Positive control siRNA (Fluorescein Conjugate)-A (sc-36869), negative control siRNA-A (sc-37007), siRNA Dilution Buffer (sc-29527), siRNA Transfection Reagent (sc-29528), siRNA Transfection Medium (sc-36868) were provided from Santa Cruz Biotechnology.

2.2. Cell culture

MIN6 mouse pancreatic beta cell line (Cell No: IBRC C10524) was provided from Iranian Biological Resource Center, and cultured in DMEM high Glucose (25 mM), 15% FBS, 70 μM mercapto-ethanol and 1% Penicillin/Streptomycin. Cells were maintained in 5% CO2 atmosphere under humidified incubation and passaged by trypsinization.

2.3. Gene expression assay by real-time quantitative PCR (RT-PCR)

A_{2a} receptor gene expression in MIN6 cells was evaluated with RT-PCR. In addition, an IPP-1 gene expression level in response to the CGS 21680 was also quantified. Briefly, MIN6 cells (300×10^3 cell/well) were seeded in the 6-well plate and incubated overnight. Then, cells were treated with CGS 21680 (0.01–10 μM) for 12 h. After extraction of total RNA and cDNA synthesis, desired genes amplicon was extended using specific primers, which is listed in the Table 1. The running protocol was included holding stage (10 min at 95 °C), cycling stage (95 °C for 15 s, 60 °C for 1 min that was repeated 45 cycle), and melting curve stage (95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s), which exerted by Applied Biosystems instrument (ABI 7500 Real-Time PCR System, Foster City, USA). The expression levels of a housekeeping gene, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were used as an internal control in order to normalize threshold cycle (Ct) of IPP-1 in each treatment. The results were calculated based on relative gene expression level, ($2^{-\Delta\Delta CT}$).

2.4. Cyclic AMP assay

3×10^5/well were seeded in 6 well plate for overnight. Then, cells were washed twice with HHBS buffer and pretreated 15 min with Ro-20–1724 (100 μM), a specific cAMP phosphodiesterase inhibitor. For A_{2a} receptor activity assessment, CGS 21680 (at concentrations between 10^{-3} and 10^{-9} M) were prepared in HHBS buffer (Solarbio life sciences) and added to the cells for 15 min in presence or absence of DMPX (1 μM). At the end of treatments, the solution was removed and the reactions were stopped immediately with ice-cold HCl 0.1 M. Before assessment, cAMP was acetylated using potassium hydroxide and acetic anhydride acid and its level was determined using competitive EIA kit. Dose response curves and effective concentration (EC_{50}) value were calculated using Graphpad Prism software.

2.5. PKA activity assay

MIN6 cells were cultured in 6-well plate to reach 90% confluency. These cells were treated with 0.01–10 μM of CGS 21680 and Forskolin 10 μM, an adenylyl cyclase agonist, for 30 min. Then, PKA activity was evaluated following to the manufacturer's instructions and by using a specific PKA activity assay kit (Abcam). Briefly, after treatment, MIN6 cells were lysed and protein content of each sample was determined with Bradford assay. 10 μg of each lysates was used for the detection of specific phospho-peptide substrate by ELISA method, which was phosphorylated by active PKA.

2.6. Gene expressions knock down by small interfering RNA (siRNA) transfection

Specific IPP-1 or negative control (with no known targetable gene) siRNAs, at final concentration 50 nM, were transfected into MIN6 cells using specific Transfection Reagent and according to the manufacturer siRNA Transfection protocol (Santa cruz). Briefly, 2×10^5 cell/well was seeded in 6 well plates without antibiotic, overnight. Transfection Reagent and siRNAs were added to the Transfection Medium and incubated for 45 min to form siRNA loaded liposomes. Then, MIN6 cells washed twice with Transfection Medium and loaded liposomes were added to each well, then plate was incubated for 7 h in CO2 incubator. Finally, Transfection Medium was removed and complete culture medium was added to plate. All treatments and assessments were performed after 72 h post transfection.

2.7. MTT assay

We cultured MIN6 cells, 5×10^3/well, overnight in 96-well plate. Then, these cells and IPP-1 knocked down cells (72 h post siRNA transfection) were treated with CGS 21680 (0.01–10 μM) in presence or absence of DMPX (1 μM) for 48 h. Next, MTT assay was performed as previously described (Hashemi et al., 2005; Bahmani et al., 2017). Briefly MTT dye, with final concentration of 5 mg/ml in PBS, was added to each well and plate incubated for 4 h to allow Formazan crystals formation. After this time, the supernatant was removed and Formazan crystals dissolved in DMSO, and the absorbance of each well was quantified at 570 nm using a Synergy H1 Multimode Microplate Reader (Biotech Instruments, Winooski, VT).

2.8. BrdU cell proliferation assay

MIN6 cells, at the 5×10^3/well concentration, were seeded 24 h prior to assessment in 96-well plates. Then, these cells and IPP-1 knocked down cells (72 h post siRNA transfection) were treated for 12 h

Table 1
Primer sequences of related genes which were used for RT-PCR.

Gene symbol	Accession number	Forward primer	Reverse primer
A2a receptor	NM_009630.3	GAGCAAGAGGCAGGTATCTCA	CAGCCCTTTCCTCACAAGAG
IPP-1	NM_001163434	GAATCCAACCCCAAGACTCA	CCAACTTCTGTCAGACCAAGC
GAPDH	NM_008084 NM_001289726	GTCGGTGTGAACGGATTTG	AGGTCAATGAAGGGGTCGT

with CGS 21680 (0.01–10 μM) and 5-bromo-2′-deoxyuridine (BrdU) (10 μM) in presence or absence of DMPX (1 μM). After, the supernatant was removed and following manufacturer instructions, Brdu assay performed. Briefly, cell lysed, DNA fixed and denatured. Next, the supernatant was drained out, detection antibody (1:1000) was added to each well and plate incubated at room temperature for 1 h. Plate was washed 3 times using wash buffer and adding of HRP-conjugated antibody to each well and incubated for another 30 min. After 3 times washing TMB substrate added to plate and incubated 30 min at room. The staining procedure was stopped with STOP solution and the absorbance measured at 450 nm with Synergy H1 Multimode Microplate Reader.

2.9. Insulin secretion assay

20×10^3 cell/well was cultured overnight in the 96-well plate. Then, these cells and IPP-1 knocked down cells (72 h post siRNA transfection) were washed three times with Krebs-Ringer (KRBH) buffer with pH 7.4 (containing: 135 mM NaCl, 1.5 mM CaCl2, 4 mM KCl, 1 mM KH2 PO4, 1.2 mM MgSO4, (pH 7.4), 2 mM NaHCO3, 10 mM HEPES, 2.5 mM D-glucose, and 1.5% bovine serum albumin free fatty acid). Then, MIN6 cells were treated with 100 μl of this buffer for 60 min. CGS 21680 at concentration between (0.01–10 μM) was provided in KRBH buffer containing 16.5 mM D-glucose and then added to each well (100 μl/well). After 60 min incubation, the supernatant were collected and content of their insulin was evaluated according to the manufacturer's instruction of sandwich based-enzyme immunosorbent assay (EIA) kit (Mercodia Mouse Insulin ELISA).

2.10. Western blot analysis

Akt, p-Akt, IPP-1, p-IPP-1, CREB, p-CREB, Bcl-2 and Beta-actin protein levels were determined with western blotting. Briefly, MIN6 cells, at 5×10^5/well were cultured in 6 well plates, overnight. Then, these cells were treated with CGS 21680. Whole protein content of treated cells was extracted using RIPA lysis buffer containing 0.5 mM PMSF, and 0.5% protease and phosphatase inhibitor cocktails, as previously described (Hashemi et al., 2007; Poupel et al., 2017). Protein content of each extract was quantified with Bradford assay and Bovine serum albumin as standard. Total protein was resolved in 12% SDS-PAGE gel and then transferred to the PVDF membrane. Protein of interest was revealed using specific primary and appropriate secondary antibodies and with a chemiluminescent substrate (ECL). Picture of each western blot band was semiquantified using Image J software and normalized to the β-actin.

2.11. Statistical analysis

Each assessment repeats were expressed at legend of figures. The statistical significance of data, with a P value < 0.05, was tested by one-way analysis of variance (ANOVA) using Statistical Package for the Social Sciences (SPSS). ED_{50} values were calculated by GraphPad Prism software. All results presented as means ± S.D.

3. Results

3.1. MIN6 cells express functional A_{2a} receptor which recruited cAMP as a secondary messenger

A_{2a} receptor expression in MIN6 cells was established using RT-PCR (Fig S1). Its mRNA expression levels in these cells were lower than A1, but were significantly higher than A2b and A3 receptors. To determine A_{2a} receptor functionality in this cell line, following specific agonist (CGS 21680) and antagonist (DMPX) treatments, cAMP concentration was quantified. According to our results A_{2a} receptor stimulation with CGS 21680 increased cAMP levels in MIN6 cells in a concentration dependent manner (Fig. 1), the EC_{50} value for CGS 21680 was calculated 2.41 μM. However, DPMX pretreatment antagonized the CGS 21680 mediated elevation of cAMP (Fig. 1). Altogether, these results indicated that A_{2a} receptor is functional in MIN6 cells and actively increased cAMP levels in response to CGS 21680 agonist. This response was abrogated with DMPX antagonist, which confirmed involvement of A_{2a} receptor in elevation of cAMP.

3.2. A_{2a} receptor agonist increases viability and proliferation of MIN6 Cells as well as potentiate GSIS

To examine effects of A_{2a} receptor stimulation on MIN6 cells, we evaluated the impact of CGS 21680 on MIN6 cell viability and proliferation by MTT and Brdu assays. According to our MTT assay results (Fig. 2A), activation of A_{2a} receptor caused to an increase in MIN6 cell viability, which was in dose-dependent manner, starting at 0.1 μM and increased up to 10 μM (from 118.97 ± 5.42% for 0.1 μM to 135.52 ± 4.3% for 10 μM). In agreement with our MTT analysis, as mentioned in Fig. 2B, Brdu evaluations also showed similar dose-dependent increasing patterns of proliferation. However, in DMPX pretreated cells (1 μM for 1 h), CGS 21680 was unable to increase both cell viability and proliferation ($p > 0.05$) (Fig. 2A and B). Altogether, these results indicate that stimulation of A_{2a} receptor by CGS 21680 increased MIN6 cell viability and proliferation. These effects of CGS 21680 were blocked by DMPX antagonist, which indicate specific involvement of

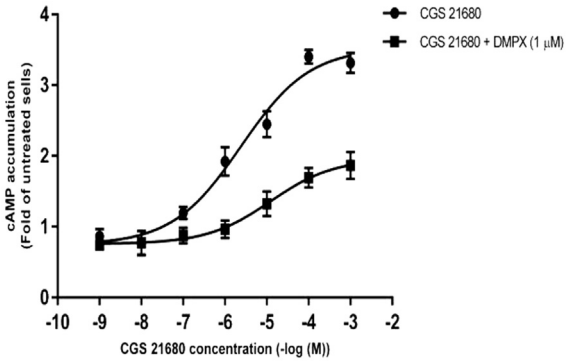

Fig. 1. A_{2a} receptor functionally increased cAMP levels in MIN6 cells. Cells were treated with different concentrations of CGS 21680 for 15 min in presence or absence of DMPX antagonist (1 μM), then, cAMP content was assayed using colorimetric competitive ELISA. This assays were duplicated twice (n = 4) and results presented as mean ± S.D.

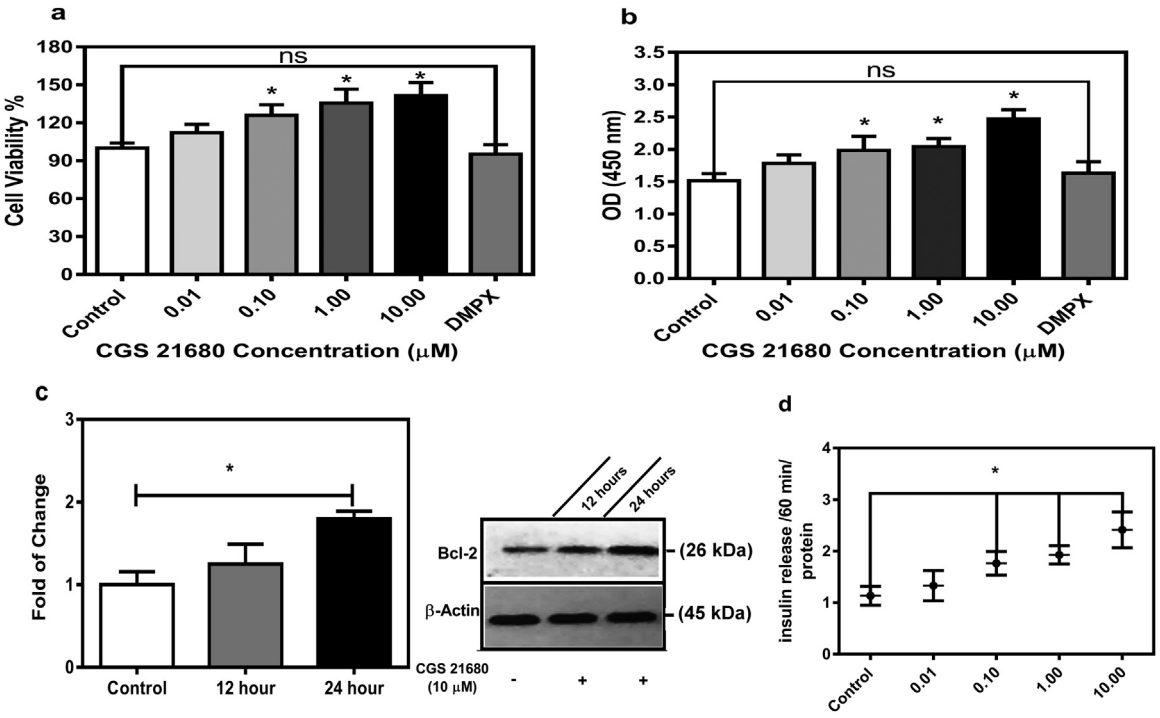

Fig. 2. CGS 21680 increases MIN6 cell viability and proliferation as well as potentiate GSIS. **A**) MIN6 cells were treated with different concentrations of CGS 21680, in presence or absence of DMPX (1 μM), for 48 h and then with MTT dye as a substrate for mitochondrial succinate dehydrogenase. This assay was triplicated in three independent times. **B**) MIN6 cells treated with CGS 21680 (0.01–10 μM) and Brdu (10 μM), in presence or absence of DMPX (1 μM), for 12 h. **C**) Time dependent increase of Bcl-2 expression in response to the CGS 21680 (10 μM). This assay was duplicated three times (n = 6) independently. **D)** MIN6 cells were treated with different concentrations of CGS 21680 for 60 min and then insulin content of supernatant was evaluated with ELISA. This assay, also, was duplicated in three independent times (n = 6). All results were presented as mean ± S.D. and P value < 0.05 (*) was considered significant, statistically. ns: not significant.

A_{2a} receptor.

The capability of A_{2a} receptor to increase of MIN6 cell viability prompted us to investigate the expression levels of prosurvival Bcl-2 protein. In this regard, we evaluated the protein levels of Bcl-2 in MIN6 cells following treatment with CGS 21680 for time intervals of 12 and 24 h. Our results (Fig. 2C) showed that stimulation of A_{2a} receptor caused an increase in Bcl-2 expression, which was 1.79 ± 0.09 fold after 24 h post treatment (compared to control, $P < 0.05$).

To determine whether A_{2a} receptor activation leads to an increase in insulin secretion by MIN6 cells, we treated these cells with CGS 21680 for 60 min and measured insulin content of supernatant with sandwich-ELISA. The results, which are presented in Fig. 2D, showed CGS 21680 at higher concentrations potentiates insulin secretion from MIN6 cells (1.95 ± 0.30 and 2.26 ± 0.15 for 1 and 10 μM, respectively).

3.3. PKA, IPP-1, CREB and Akt are the mediators of A_{2a} receptor signaling in MIN6 cells

PKA activity, IPP-1, CREB and Akt phosphorylation levels have been investigated to evaluate signaling cascade of A_{2a} receptor pathway. Accumulation of cAMP in response to CGS 21680 (Fig. 1) prompted us to evaluate PKA activity as a downstream signaling mediator of cAMP. According to our results, following CGS 21680 treatment, relative activities of PKA (Fig. 3A) was increased in a concentration-dependent manner (trending from 0.61 ± 0.06 for 0.1 μM to 0.79 ± 0.018 for 10 μM). These findings indicate that part of A_{2a} receptor signaling pathway mediated through activation of PKA.

IPP-1 is abundantly and selectively expressed in human or rodent PBCs. Its protein levels was estimated 20% of housekeeping protein, GAPDH (Jiang et al., 2013). Active PKA directly phosphorylate IPP-1 at Thr-35 residue (Aggen et al., 2000). According to our Western blotting results, CGS 21680 increased p-IPP-1 levels in a dose- and time-dependent manner (Fig. 3B and C). Maximum levels of these phosphorylation were observed at 10 μM of CGS 21680 (3.39 ± 0.98 fold) and 30 min (12.5 ± 0.948 fold) after treatment of MIN6 cells with this concentration, in comparison with untreated cells, $P < 0.01$).

It has been established that signaling cascade through Akt is essential for normal PBCs function and proliferation (Bernal-Mizrachi et al., 2004). Thus, to further identify the master regulators of A_{2a} receptor signaling in MIN6 cells, we next analyzed Akt phosphorylation status using western blot assay. As shown in Fig. 3b and c, incubation with CGS 21680 increased p-Akt level in the dose and time dependent manner with maximum effects at 10 μM and 30 min after CGS 21680 treatment (14.13 ± 0.57 and 11.24 ± 0.94 fold vs. control group, $P < 0.01$, respectively).

CREB is one of the main mediators that is participates in insulin biosynthesis and survival of PBCs (Shin et al., 2014). Thus, to determine whether CREB mediates these effects of A_{2a} receptor on MIN6 cells, following CGS 21680 treatments, its phosphorylation level was analyzed. The results indicated that CREB phosphorylation level (Fig. 3B and C) increased in the dose- and time-dependent manner with the maximum level at 10 μM and 30 min after CGS 21680 treatment (5.48 ± 0.3 and 5.52 ± 0.38 fold in comparison with untreated cells, $P < 0.01$, respectively).

Altogether, these assessments revealed that A_{2a} receptor activates some of main signaling branches through PKA and Akt. These signaling leaded to increased p-IPP-1/IPP-1 and p-CREB/CREB levels.

3.4. Increase in viability and proliferation of MIN6 cells as well as potentiation of insulin secretion by A_{2a} receptor is dependent to IPP-1

We indicated that IPP-1 phosphorylated following A_{2a} receptor stimulation, however, its role in PBCs and essentiality of its presence in A_{2a} receptor signaling pathway are not cleared yet. Thus, to deals with these issues, we determined IPP-1 levels in response to the A_{2a} receptor activation by RT-PCR and western blot. According to our results, after

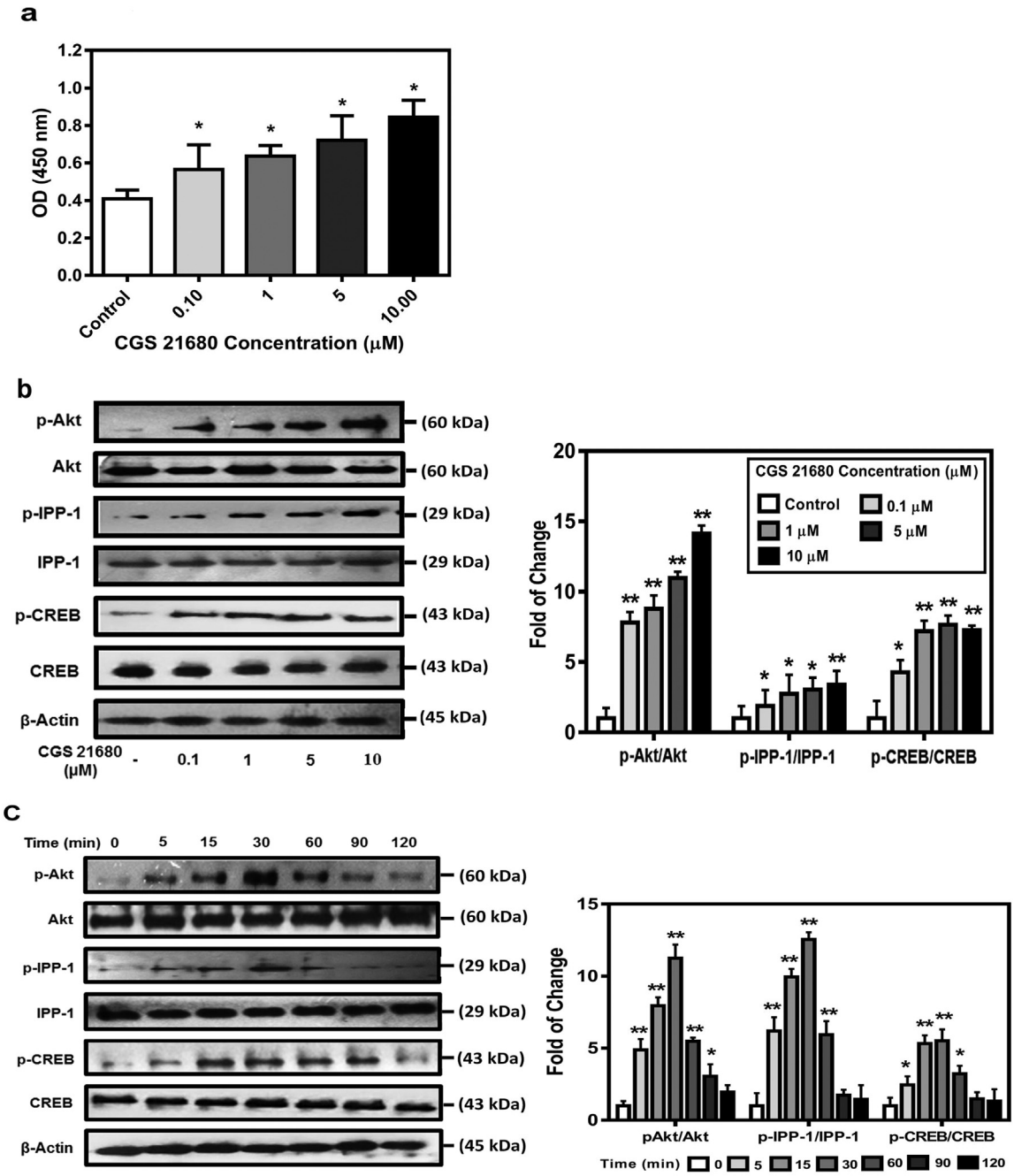

Fig. 3. PKA, IPP-1, CREB and Akt activated in dose and time dependent manner in response to A_{2a} receptor stimulation by CGS 21680 (10 μM). (**A**) Cells were cultured overnight and then treated with different concentration of CGS 21680 for 30 min. Total protein was extracted and PKA activity assayed with specific ELISA kit. (**B**) MIN6 cells were treated with CGS 21680 for different concentrations (0.1–10 μM) for 15 min and then (**C**) 10 μM was chosen to determine phosphorylation status of Akt, IPP-1, and CREB in a time interval manner (0, 5, 15, 30, 60, and 120 min) using Western blot. This assays were duplicated three time (n = 6) independently. Results were presented as mean ± S.D., $P < 0.05$ (*) and $P < 0.01$ (**) were considered significant.

12 h treatment with different concentration of CGS 21680, IPP-1 mRNA was upregulated (Fig. 4A). IPP-1 upregulation was dose dependent and has significant increase from 1 μM to 10 μM, 1.6 ± 0.21 fold to 1.9 ± 0.13 in comparison with untreated cells, respectively. CGS 21680 at 10 μM effectively caused IPP-1 overexpression, thus, this concentration was chosen and protein levels of IPP-1 were evaluated at time dependent manner. As mentioned at Fig. 4B, IPP-1 protein level was increasing up (P value < 0.05), 1.67 ± 0.084 fold after 24 h post treatment with CGS 21680 (10 μM), in comparison with untreated cells. Collectively, these results showed that A_{2a} receptor activation by CGS 21680 increased IPP-1 expression at both mRNA and protein levels.

To evaluate the essentiality of IPP-1 in A_{2a} receptor signaling dependent increases of MIN6 viability, proliferation and insulin secretion, we used IPP-1 siRNA to knock down its expression in MIN6 cells, then, the effects CGS 21680 were assayed on survival and insulin secretion. IPP-1 knocked down efficiency by siRNA in both mRNA and protein levels was evaluated by RT-PCR and western blotting, 72 h after transfection. As depicted in Fig. 4C, IPP-1 mRNA levels was decreased 74.7% and 76.85%, in comparison with control and scramble, respectively. IPP-1 protein levels also decreased to 75.7% and 77.18% of control and scramble (Fig. 4D), respectively. There is no significant alteration in IPP-1 expression between control and scramble groups,

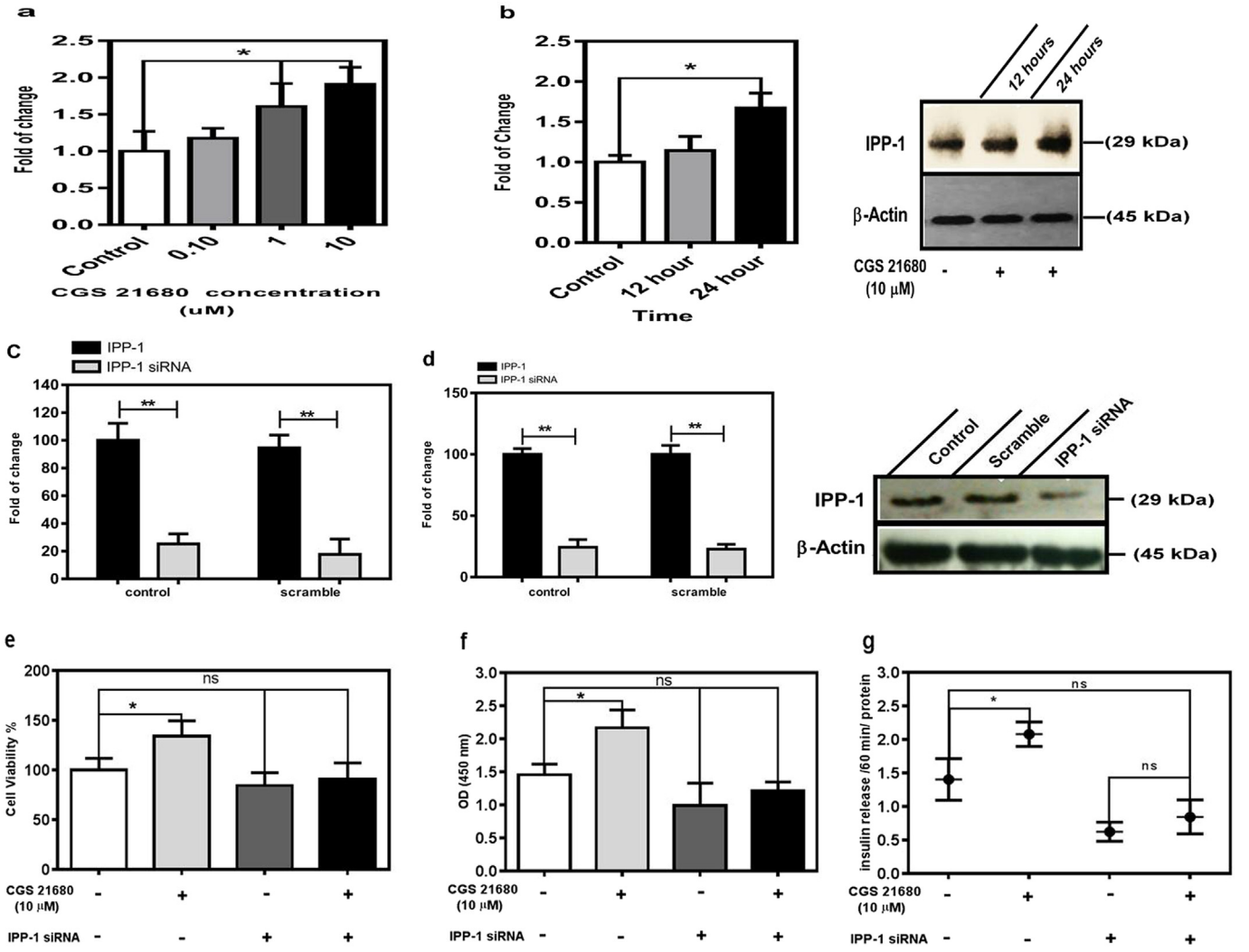

Fig. 4. A_{2a} receptor is dependent to IPP-1 in order to increase MIN6 cells proliferation and potentiate insulin secretion. **A)** MIN6 cells were treated with different concentrations of CGS 21680 for 12 h and then IPP-1 mRNA level was determined with RT-PCR (n = 9). **B)** Cells treated with CGS 21680 (10 μM) for indicated times and IPP-1 protein level was determined with western blot. Then, cells were treated with 50 nM of IPP-1 and scramble siRNA and IPP-1 expression was assayed with (**c**) RT-PCR (n = 6) and (**D**) western blot (n = 6), 72 h post transfection. RT-PCR and immunoblotting data were normalized with GAPDH and Actin, respectively. Again, cells were transfected with dedicated siRNAs and their (**E**) viability, (**F**) proliferation and (**G**) insulin secretion were assayed 72 h post transfection. These assays were duplicated three times (n = 6) independently. Each blot was semiquantified using Image J software. All results were presented as mean ± S.D. and P value < 0.05 (*) and < 0.01 (**) was considered significant, statistically. ns: not significant.

$P < 0.05$.

According to our MTT and Brdu results (Fig. 2) 10 μM of CGS 21680 is more effective on stimulation of MIN6 viability and proliferation, thus this concentration was chosen for IPP-1 knock down assays. IPP-1 knocked down MIN6 cells were treated with 10 μM of CGS 21680 and their viability and proliferation were evaluated with MTT and Brdu assays, after 48 h and 12 h, respectively. According to our results, A_{2a} receptor failed to increase viability and proliferation of these cells (Fig. 4E and F), $P < 0.05$. In addition, insulin secretion was not showed a significant increase following CGS 21680 treatment ($P < 0.05$) (Fig. 4G). These results indicated that IPP-1 is required for A_{2a} receptor mediated increase in viability, proliferation and insulin secretion of MIN6 cells.

3.5. IPP-1 controls signaling extension of A_{2a} receptor in MIN6 cells

To further focus on IPP-1 role in A_{2a} receptor signaling pathway, we used an IPP-1 siRNA to investigate CREB and Akt phosphorylation levels in response to the CGS 21680 (10 μM). MIN6 cells (scramble or IPP-1 siRNA transfected) treated for 30 min with this agonist and then subjected to immunoblotting for detection of p-Akt and p-CREB levels. We found that IPP-1 knock down robustly decreased p-(Ser133)–CREB and p-(Ser473)-Akt levels in response to CGS 21680 (10 μM) (Fig. 5). Based on these findings, we propose that IPP-1 knock down in MIN6 cells rescued PP-1, which leads to disruption of kinases/phosphatases balance (Fig. 6).

4. Discussion

Most of therapeutic interventions for management of hyperglycemic conditions are supportive (Chaudhury et al., 2017) and limited progress has been made to directly increase PBCs proliferation. Signaling through several receptors, such as: GLP-1 and IGF-1 (Chen et al., 2009), appears to increase PBCs mass. However, because of many limitations, usefulness of these receptors is arguable. For example: exenditn-4 as a GLP-1R peptic agonist, increases potential risk for the pancreatitis, pancreatic and thyroid cancers (Butler et al., 2013) and activation of IGF-1 signaling in PBCs do not efficiently increase PBCs mass

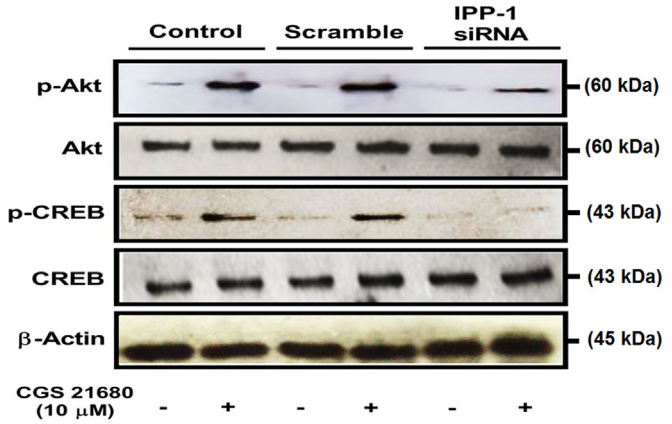

Fig. 5. A_{2a} receptor signaling activation failed to increase CREB and Akt phosphorylation in IPP-1 knocked down MIN6 cells. These cells were treated with CGS 21680 (10 μM) for 15 min and phosphorylation levels of Akt, and CREB were determined using Western blotting. These assays were duplicated three times (n = 6) independently.

(Andersson et al., 2012). Recently, it has been reported that activation of A_{2a} receptor potently and specifically increases PBCs mass (Andersson et al., 2012; Ohtani et al., 2013). Herein, we investigated intracellular signaling mediators of this receptor in MIN6 cells and mentioned that activation of this receptor resulted in increase of PKA activity, p-Akt, p-IPP-1, and p-CREB levels. Moreover, we highlighted pivotal role of IPP-1 in extension of A_{2a} receptor signaling in these cells. Identification of key mediators of A_{2a} receptor signaling pathway give new mechanistic insights into engaged pathways in PBCs proliferation and may open new rational opportunities for remedial purposes.

Previous studies showed A_{2a} receptor highly expressed in Zebrafish and mouse islets (Schulz et al., 2016) or even in βTC-6 cell line (Ohtani et al., 2013). We also confirmed that MIN6 cell line express this receptor. In this cells, CGS 21680 agonist actively increased cAMP levels, which antagonized with DMPX. This pharmacological profile implicated specific involvement of A_{2a} receptor. In addition, DMPX inhibited survival, proliferative and insulin secretion activity of CGS 21680. Previous studies highlighted cAMP/PKA signaling in PBCs (Jhala et al., 2003; Xie et al., 2007). The fact that A_{2a} receptor increase insulin secretion and proliferation of MIN6 cells were in agreement with in vivo studies that showed stimulation of PBCs and increased islet mass (Andersson et al., 2012; Schulz et al., 2016; Csóka et al., 2017). However, M. Ohtani and et al. showed treatment of βTC-6 cell line with CGS 21680 has no proliferative effect and even mildly decreased their viability (Ohtani et al., 2013). MIN6 cell line is more differentiated than βTC-6 and shows similar features to human and mouse PBCs (Ishihara et al., 1993; Rosengren et al., 2012). Even insulin secretion responses to elevated Glucose levels are generally different between these lines (Skelin et al., 2010). Thus, the contradictory effects of CGS 21680 agonist on MIN6 and βTC-6 cells may be related to the different characteristic features of these cell lines.

Herein, we mentioned that CGS 21680 increased p-Akt levels in MIN6 cells. Effects of A_{2a} receptor activation on p-Akt levels, however, appear to be cell type specific. For example, these effects may be reflected as increase (in PC12 (Mori et al., 2004) and pulmonary endothelial cells (Ahmad et al., 2013)), decrease (in BRA-2 (Ke et al., 2009) and naïve T cells (Cekic et al., 2013)), or no effects (in rat mast cells (Gao et al., 2001) and porcine coronary artery smooth muscle cells (Shen et al., 2005)). Akt deficient mice manifested with insulin resistance and PBC failure, which indicate critical essentiality of Akt in PBCs biology. Its signaling in PBCs attributed to proliferation, neogenesis, insulin secretion and control of cell size (Elghazi et al., 2006). Thus, it is possible that A_{2a} receptor, by activation of Akt signaling pathway, profoundly adapt PBCs to pathophysiological conditions. However, it should be mentioned that A_{2a} receptor signaling mostly increases PBCs proliferation not neogenesis (Andersson et al., 2012). GLP-1 receptor also activates Akt in INS-1 cells (Wang et al., 2004), however, its agonist (exendin-4) increases both proliferation and neogenesis of PBCs (Xu et al., 1999).

Both PKA (Jhala et al., 2003) and Akt (Ghavami et al., 2009; Li et al., 2011) induced phosphorylation of CREB at ser-133. Our results indicated that A_{2a} receptor activation increased p-(Ser133)–CREB levels in dose and time dependent manner. At least in PBCs, CREB exclusively is required for regulated expression of insulin and antiapoptotic proteins like Bcl-2 (Dalle et al., 2011). In this study we showed that A_{2a} receptor stimulation with CGS 21680 mildly increased Bcl-2 expression, possibly through p-CREB. Bcl-2 antiapoptotic protein is one of main regulators of MIN6 and β-cells viability and function (Luciani et al., 2013). Of note, Bcl-2 overexpression may interrupt PBCs response to

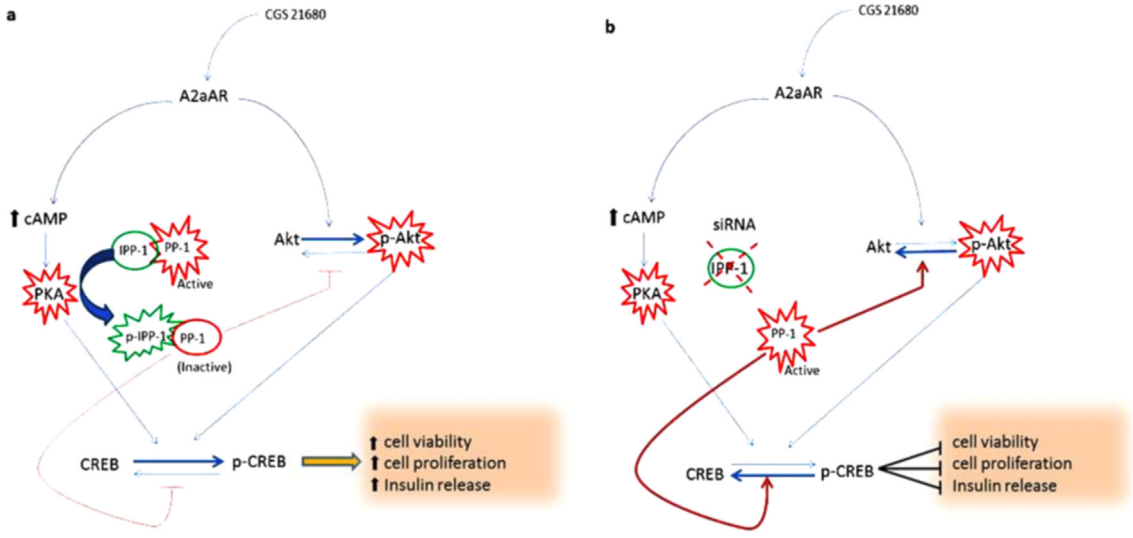

Fig. 6. Possible signaling pathways of A_{2a} receptor in MIN6 cells in absence (**A**) or presence (**B**) of IPP-1 siRNA. PKA, through IPP-1 phosphorylation, most likely controls PP-1 activity. At this condition, deactivation of PP-1 allowed p-Akt and p-CREB to increase MIN6 viability, proliferation, and insulin releases. IPP-1 appears to act as a key regulator of phosphorylation/dephosphorylation balance in MIN6 cells. Highlighted arrows indicated for predominant reactions in normal (**A**) or IPP-1 knocked down cells (**B**). For further information, see text.

the Glucose and prevent augmentation of insulin production (Luciani et al., 2013). Indeed, decreased insulin production is beneficial for β-cell viability and proliferation (Szabat et al., 2016), which lowers secretory burden in these cells. However, we mentioned that CGS 21680 increased insulin secretion from MIN6 cells. It should be considered that acute exposure (1 h) of MIN6 cells with CGS 21680 augmented insulin secretion. However, Bcl-2 overexpression was obvious after 12 h treatment.

The exchange protein directly activated by cAMP (Epac) signaling also mediate part of cAMP signaling in PBCs (Holz, 2004). However, Shibasaki showed that the actions of cAMP on insulin release can be blocked by PKA inhibitor (H89), but not mimicked by the Epac activator (8-CPT-20–O-Me-cAMP) (Shibasaki et al., 2007). Although, Epac mostly enforce fast release of insulin through increase of intracellular Ca^{2+} levels (Holz, 2004), but, it appears that role of PKA in the first phase of insulin exocytosis is more highlighted than Epac (Renström et al., 1997; Hatakeyama et al., 2006). Moreover, previous studies mentioned that 8-CPT-20–O-Me-cAMP has no effects on CREB phosphorylation (Shibasaki et al., 2007; Chepurny et al., 2009). However, it is possible that Epac also play significant role in A_{2a} receptor signaling cascade to mediate insulin secretion.

In MIN6 cells upon 10 min after Glucose treatment phospho-protein levels significantly increased to induce insulin secretion. Moreover, recent evidences revealed profound changes of kinases and phosphatases balance in prediabetic and diabetic patients (Sacco et al., 2016). In these patients IPP-1 expression also decreased, which highlights its possible role in pathogenesis of diabetes. Our study revealed that IPP-1 expression was increased upon induction of MIN6 proliferation by CGS 21680. It has been shown that short term exposure of human islets to high Glucose also increases IPP-1 expression (Taneera et al., 2014). This overexpressed IPP-1 probably helps MIN6 cells to have better control on Phosphorylation/dephosphorylation balance. In addition, it has been shown that in MIN6 cells, IPP-1 form a complex with PP-1 and eIF2α, which enables instant dephosphorylation of eIF2α to increase general translation in response to elevated Glucose levels (Vander Mierde et al., 2007). IPP-1 knockdown appears to profoundly disrupted CGS 21680 effects on insulin secretion. According to our results, knock down of this protein vigorously decreased p-Akt and p-CREB levels (Fig. 9). IPP-1 is specific regulator of PP-1 and its activation by PKA appear to has no effects on other protein phosphatases (Weiser et al., 2004). Thus, we propose that disruption of Akt and CREB phosphorylation by A_{2a} receptor agonist maybe related to unleash of PP-1 (Fig. 6). In Jurkat cells it has been shown that p-(Ser473)-Akt is a direct PP-1 substrate (Thayyullathil et al., 2011). In addition, immunoprecipitation studies demonstrated that Akt form a complex with PP-1, which indicate sophisticate control of Akt activity (Xiao et al., 2010; Thayyullathil et al., 2011). It has been shown in granulosa cells PKA increase p-(Ser473)-Akt levels through IRS-1 and GAB2 adaptor proteins (Hunzicker-Dunn et al., 2012). Herein, we unraveled an underestimate IPP-1 dependent pathway in MIN6 cells which interconnect PKA and Akt signaling pathways. In addition, we mentioned that A_{2a} receptor activation failed to increase p-(Ser133)–CREB levels in IPP-1 knocked down MIN6 cells. CREB is the main PKA and Akt mediators that its activity appears to be necessary for survival and insulin release. This transcription factor is also one of main PP-1 substrate (Yan et al., 2016) and IPP-1 knock down possibly unleash this phosphatase and interrupt with CREB activity. Consistent with our results, in vivo studies demonstrated that transgenic mice carrying A-CREB (dominant inhibitor of CREB) fail to increase insulin release and PBCs growth and survival in response to exendin-4 (GLP-1 receptor agonist) (Jhala et al., 2003).

Recent study indicated that consistent activation of IPP-1 increases last long phospho-protein levels, which is associated with overgrowth of tumor cells (Luo et al., 2018). Moreover, role of IPP-1 in control of Phosphorylation rate in PBCs may be restricted to many signaling pathways. In most recent study, it has been mentioned that IPP-1 and PP-1 did not control KAP-1 and γH2AX phosphorylation levels following activation of DNA damage response (DDR) signaling pathway (Oleson et al., 2018). However, there is a consensus idea that PKA mediates phosphorylation of IPP-1 at threonine-35 to inhibit PP-1 activity (IC_{50} ~ 1 nM) (Endo et al., 1996). The data shown here mentioned that A_{2a} receptor activates PKA in response to the CGS 21680. Thus, it is possible that in β-cells activation of related GPCRs which coupled to induction of cAMP and PKA leads to similar effects on IPP-1 dependent control of phosphorylation levels.

5. Conclusion

In summary, our study revealed that A_{2a} receptor increase cAMP levels and recruit PKA and Akt to stimulate MIN6 cell proliferation and insulin secretion. In addition, herein, we showed that CGS 21680 treatment increased p-CREB levels which indicate that this transcription factor is possibly contribute to the A_{2a} receptor mediated regulation of gene expression. SiRNA knock down assessments suggests that IPP-1 is a key factor that control signaling extension of A_{2a} receptor in MIN6 cells. In this regards, we highlighted p-IPP-1 interconnections with p-CREB and p-Akt which affects physiological response of MIN6 cells to A_{2a} receptor stimulation (Fig. 9). Unraveling of these interconnections provides interesting opportunities for therapeutic purposes to prevent PBCs failure and increase of insulin secretory function.

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Conflict of interest

The authors declare that these are no conflict of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejphar.2019.02.017.

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