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Original Article

A3 receptor agonist, Cl-IBMECA, potentiate glucose-induced insulin secretion from MIN6 insulinoma cells possibly through transient Ca^{2+} entry

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Abstract

Diabetes incidence showed ascending trends in recent years indicating urgent need for new therapeutic agents. Extracellular adenosine signaling showed promising results. However, role of its A3 receptor in pancreatic β-cells proliferation and insulin secretion is not well established. Thus, we aimed to determine its main signaling mediators in MIN6 insulinoma cell line. A3 adenosine receptor (A3AR) expression was confirmed using RT-PCR. Receptor functionality was evaluated by measurements of cAMP, using ELISA kit, and intracellular Ca^{2+} levels, using Fura 2/AM probe in response to the specific A3AR agonist (Cl-IBMECA). Insulin ELISA kit was used to measure insulin release. Herein, we mentioned that MIN6 cells express active form of A3AR, which decreased cAMP levels with the half maximal effective concentration (EC50) value of 5.61. $[Ca^{2+}]i$ Levels transiently (approximately 120 sec) increased in response to the agonist. Cl-IBMECA increase insulin secretion at 0.01-1 μM, but showed an inhibitory effects at higher concentrations (1-10 μM). Altogether, we found that in MIN6 cells, A3AR, possibly through Ca^{2+} mediated signaling pathways, potentiated glucose-induced insulin secretion.

Keywords: Adenosine receptor; Cell viability; Diabetes, Insulin; MIN6 cells..

INTRODUCTION

The global statistics in 2017 estimated that there are 451 million people suffering from diabetes and hyperglycemia. In this year, diabetes was direct underlying cause of 5 million deaths. Unfortunately, expectations for future did not show any declining trends in diabetes incidence (1). In this regards, needs for finding new therapeutic agents and targets appears to be urgent.

In the last decade, more attentions have been attributed to the adenosine signaling pathways to promote pancreatic β-cells (PBCs) proliferation and potentiate insulin secretion (2-5). One of the potent mitogens of PBCs, 5'-N-Ethylcarboxamidoadenosine (NECA), activate extracellular adenosine receptors (AR) and alleviate hyperglycemic conditions (3).

Extracellular adenosine receptors subdivided into four G protein-coupled receptors (GPCRs) including: A1AR, A2aAR, A2bAR, and A3AR. All of these receptors expression were confirmed in islets and many insulinoma cell lines (2,6). Except for A3AR, effects of other adenosine receptors in PBCs biology have been widely studied. For instance, it has been shown that adenosine through activation of this receptor A1AR inhibits glucose-stimulated insulin secretion (GSIS) (7). In βTC3 insulinoma cell line and in mice with type-1 diabetes, it was mentioned that activation of this receptor has no significant effects on PBCs survival (6,8). Recent findings profoundly addressed critical roles of A2aAR in PBCs biology, including either proliferation or GSIS (8). PBCs in A2aAR ablated mice showed a reduced proliferation during pregnancy (9) and in high fat diet (HFD)-induced metabolic disorder (10). A2bAR appears to have similar effects of A2aAR in PBCs biology (11). There are many evidences which suggest a protective role for A2bAR in PBCs biology during streptozotocin (STZ)-treated mice (8).

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Despite of well-defined effects of A1AR, A2aAR, and A2bAR, roles of A3AR in PBCs are poorly understood (12,13). Even, there is a debate about its expression in islets of Langerhans (6,8). Of course, Ohtani *et al*. reported that its agonist at high concentrations (100-300 μM) increase GSIS from isolated mice islets (6).

Depending on which signaling pathway activated in cells, A3AR stimulation may lead to different cellular responses, including apoptosis, cytoprotective, and anti-inflammatory (14,15). These signaling pathways may include Gi or Gq activation of RhoA factor and phospholipase D (16), phosphorylation or even dephoshphorylation of extracellular signal-regulated kinases 1/2 (6,17) and protein kinase B (Akt) (15).

Understanding of intracellular mediators of A3AR is essential for explaining of its effects in MIN6 cells biology. To the best of our knowledge, main signaling mediators (cAMP and Ca^{2+}) of this receptor in PBCs have not been determined, yet. Investigation of these mediators may provide new insight about control of GSIS by this receptor. Thus, in this study, we aimed to evaluate main mediators of A3AR signaling in MIN6 cells.

MATERIAL AND METHODS

Chemicals

Ro-20-1724 (a phosphodiesterase inhibitor, Cat: 557502) and MRS 21680 (an A3AR antagonist, Cat: M228) were purchased from Sigma-Aldrich-Merck company (Germany). 1-[2-Chloro-6-[[(3-iodophenyl)methyl]amino]-9H-purin-9- yl] -1-deoxy-N-methyl- β-D- ribo-furanuronamide (Cl-IBMECA, an A3AR agonist, Cat: 1104) was purchased from Tocris Bioscience company (UK). Fura 2/AM (CAS 108964-32-5) was provided from Santa Cruz (USA). Forskolin (Cat: ALX-350-001) and probenecid (Cat: ALX-430-113) were from Enzo Life Sciences. Cell culture supplies like the Dulbecco's modification of Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin/streptomycin were from Gibco Life Sciences (USA). Cyclic AMP ELISA Kit (Cat: 581001) from Cayman chemical was provided (Michigan, USA). Mouse insulin ELISA kit (Cat: 10-1247-01) was purchased from Mercodia (Sweden).

Cell culture

MIN6 insulinoma cell line was purchased from Iranian Biological Resource Center (IBRC) (Cell No: IBRC C10524) and maintained in DMEM high glucose (25 mM), 15% FBS, 70 μM mercaptoethanol and 1% penicillin/streptomycin under 5% CO2 and humidified atmosphere. MIN6 cells were passaged 10 times and then all assessments were performed.

Gene expression assay of A1AR and A3AR

Cells (5×10^5) were lysed to extract total RNA using RNX plus kit (Sinacolon, I.R. Iran). After cDNA synthesize, A1AR and A3AR amplicons were amplified using the following specific primers, forward 5′-GGTACAAGACAGTGGTGACTCAG-3′ and reverse 5′-AGGTTGTTCCAGCCAAACAT-3′ for A1AR, forward 5′-CCTGTGTGCTGCTGATCTTC-3′ and reverse 5′-TGAGTGGTAACCGTTCTATATCTGA-3′ for A3AR, and forward 5′-GTCGGTGTGAACGGATTTG-3′ and reverse 5′-AGGTCAATGAAGGGGTCGT-3′ for GAPDH (as an internal control). The heating protocol was included in holding (10 min at 95 °C), cycling (95 °C for 15 sec, 60 °C for 1 min, repeated for 45 cycle), and melting curve stages (95 °C for 15 sec, 60 °C for 1 min, and 95 °C for 15 sec). Applied Biosystems instrument (ABI 7500 Real-Time PCR System, Foster City, USA) was used for extension of desired amplicons. The results were calculated as relative gene expression level, ($2^{-\Delta\Delta CT}$), using the following equations.

$$\Delta CT \text{ (A1 receptor)} = (CT_{(A1)} - CT_{(GAPDH)}) \quad (1)$$

$$\Delta CT \text{ (A3 receptor)} = (CT_{(A3)} - CT_{(GAPDH)}) \quad (2)$$

$$\Delta\Delta CT \text{ (For A1 receptor)} = \Delta CT \text{ (A1 receptor)} - \Delta CT \text{ (A1 receptor)} = 0 \text{ (as control)} \quad (3)$$

$$\Delta\Delta CT \text{ (For A3 receptor)} = \Delta CT \text{ (A3 receptor)} - \Delta CT \text{ (A1 receptor)} \quad (4)$$

Cyclic AMP assay

Cyclic AMP was assayed according to the previously published protocol (18). 3×10^5 Cells/well was seeded in 6 well plate, overnight. Then, cells were pretreated

with a selective inhibitor of cAMP-specific phosphodiesterase, Ro-20-1724 (100 μM) for 15 min. Then, a specific adenylyl cyclase activator, forskolin (10 μM), was added to each well and plate incubated for 10 min. Finally, cells were treated with Cl-IBMECA (0.1-100 μM) for 15 min. After, according to the manufacturer's instruction, the cells were lysed using ice-cold HCl (0.1 M). cAMP content of each supernatant was acetylated using KOH and acetic anhydride acid and assayed with specific competitive enzyme immunoassay (EIA) kit. The half maximal effective concentration (EC_{50}) for Cl-IBMECA was estimated using Graphpad Prism software.

Measurement of intracellular calcium

Intracellular calcium oscillations were measured according to the previously published protocol (19). 5000 Cells/well were seeded in the specific florescence clear bottom 384 well plate (PerkinElmer company, Cat: 6007460), overnight. Then, cells were loaded with Fura 2/AM probe (final concentration of 5 μM, which was prepared at loading buffer containing NaCl 135 mM, KCl 2.5 mM, CaCl2 2 mM, MgCl2 2 mM, glucose 10 mM, (4 - (2 - hydroxyethyl) - 1 - piperazineethane-sulfonic acid) (HEPES) 5.5 mM, probenecid 2.5 mM, and 0.04% (w/v) of the nonionic detergent pluronic F-127, pH 7.4). The loading condition was 30 min at 37 °C and another 30 min at room temperature. Next, cells were washed three times with Hank's balanced salt solution (HBSS) buffer. The quenching assay with MnCl2 (500 mM) was employed to evaluate Fura2/AM loading efficiency. Cells stimulation was performed with Cl-IBMECA (10 μM in HBSS buffer) and fluorescence intensity was recorded at two excitation/emission 340/510 and 380/510 nm with intervals of 3 sec using Synergy H1 multimode microplate reader. Results were calculated as the ratio of the fluorescence intensity in 340/380.

Insulin secretion assay

Cells (20×10^3 /well) were seeded in 96-well plates, overnight. Next, cell culture medium was replaced with Krebs-Ringer HEPES (KRBH) buffer (Solarbio Life Science, China) which supplemented with 2.5 mM D-glucose. After 60 min incubation, this buffer was again replaced with new KRBH buffer containing 16.5 mM D-glucose and different concentrations of Cl-IBMECA (0.01-10). To confirm specific activation of A3AR by this agonist, cells were pretreated with MRS 1220 (1 μM) for 1 h prior Cl-IBMECA treatment. Then, following 60 min incubation, the supernatants were collected and their insulin content was quantified using specific ELISA assay and according to the manufacturer's instruction.

Statistical analysis

One-way analysis of variance (ANOVA) test was used to evaluate differences between control and treatments. Statistical Package for the Social Sciences (SPSS) was used to calculate *P* values. $P < 0.05$ was considered statistically significant.

RESULTS

MIN6 cells express A3 adenosine receptor

To evaluate gene expression of A3AR and A1AR in MIN6 cells, we extracted total RNA from these cells. Then, cDNA was synthesized and specific amplicons were extended using qRT-PCT. We also amplified amplicon of A1AR in order to compare A3AR expression levels with this receptor. Amplification plot of related genes, as well as GAPDH, are presented in Fig. 1a and indicating cycle by cycle events in qRT-PCR reactions. Melt cure plot also has been embedded in the Fig. 1b, indicating specific annealing of designed primers.

According to our results (Fig. 1c), MIN6 cells express A3AR mRNA. However, its expression level was lower than A1AR. In comparison with A1AR receptor, 0.14 ± 0.098 fold of changes was found for A3AR. Altogether, these results confirmed expression of this receptor in MIN6 cells.

A3 adenosine receptor actively decrease intracellular cAMP levels in MIN6 cells

To confirm A3AR is active in MIN6 cells, we first pretreated these cells with forskolin for 10 min. This pretreatment is necessary

because previous studies indicated A3AR is a Gi/o coupled receptor and decrease cAMP levels following stimulation (20). Next we measured cAMP levels in response to specific A3AR agonist, Cl-IBMECA. As illustrated in Fig. 2, Cl-IBMECA, in a dose dependent manner, decreased forskolin-induced cAMP levels. The EC_{50} value for this agonist was calculated to be 5.61 µM with Graphpad prism software. Altogether, these results implicated that MIN6 cells expresses an active form of A3AR, which is able to decrease cAMP levels upon stimulation with specific Cl-IBMECA agonist.

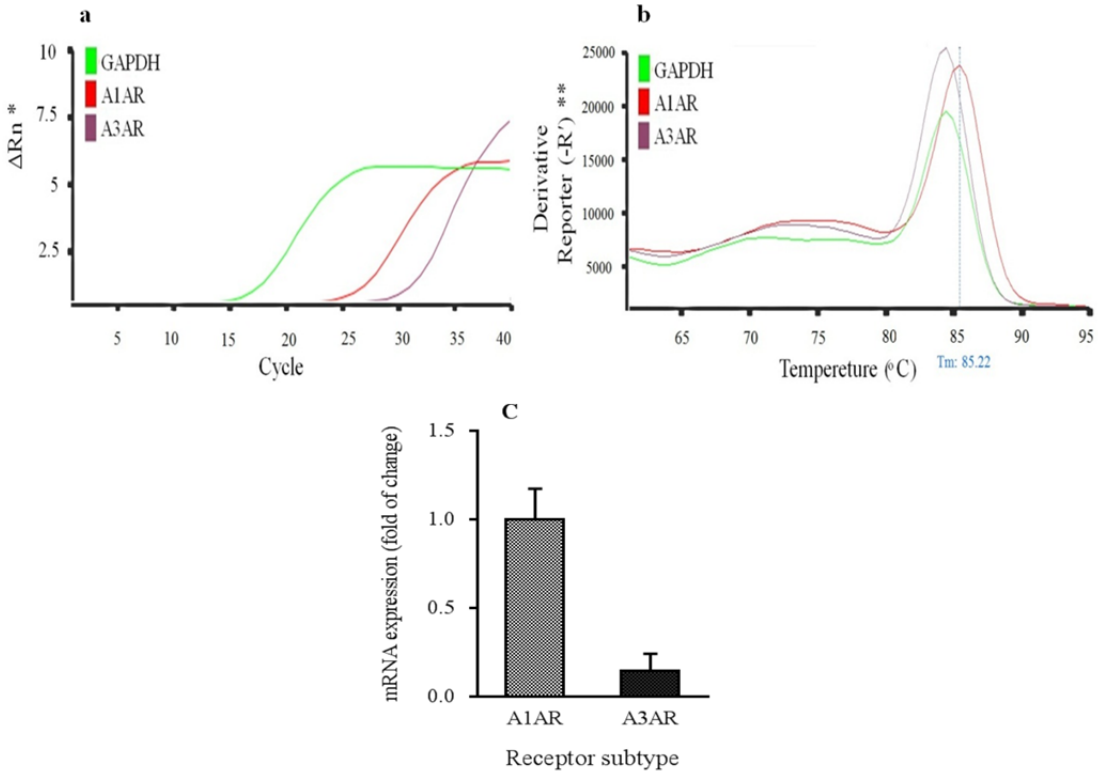

Fig. 1. MIN6 cells express A3AR. Total RNA was extracted from MIN6 cells and after cDNA synthesis specific amplicon of A3AR was extended using RT-PCR. (a) Amplification plot in linear form, (b) melt curve, and (c) gene expression levels of A3AR, in comparison with A1AR are presented. Comparison between A1 and A3 receptor expression levels was performed based on $2^{-\Delta\Delta CT}$ formula. This assay was duplicated in three independent time (n = 6). (*) ΔRn is the value obtained from subtracting of Rn from the baseline: (ΔRn = Rn - baseline), where Rn is the ratio of the emitted fluorescence intensity of the Sybr green to the emitted fluorescence intensity of the passive reference dye (ROX). (**) Derivative reporter (-Rn′) is the maximum rate of fluorescence changes following the ramping temperature. A3AR, A3 adenosine receptor.

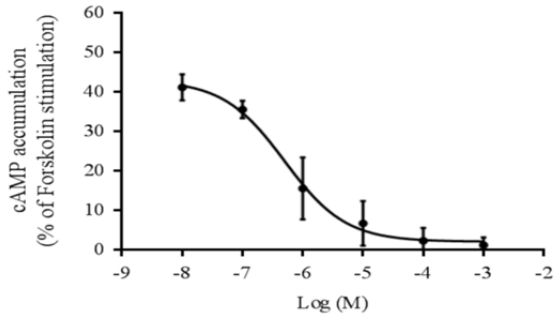

Fig. 2. MIN6 cells express an active form of A3AR. Cells were pretreated with Ro-20-1724 (100 µM) for 15 min and then, forskolin (10 µM) for 10 min. Next, Cl-IBMECA (0.1-100 µM) was added to cells for 15 min. Then, cAMP content of each treatment was assayed using specific ELISA. EC_{50} value was calculated using Graphpad Prism software. This assay was duplicated three times (n = 6) and results were presented as mean ± SD. A3AR, A3 adenosine receptor; Ro-20-1724, a phosphodiesterase inhibitor; Cl-IBMECA, A3AR agonist; EC_{50}, the half maximal effective concentration.

A3 adenosine receptor elevates intracellular Ca^{2+} levels in MIN6 cells

In addition to Gi/o coupling and decrease of intracellular cAMP levels, A3AR may be modulate $[Ca^{2+}]_i$ levels through Gq proteins (15). To address this possible effect of A3AR in Ca^{2+} modulation, we treated MIN6 cells with $[Ca^{2+}]_i$ probe (Fura 2/AM) and then stimulated with Cl-IBMECA agonist. Inside the cells Fura2/AM can be converted to the active form, Fura 2, which is able to bind $[Ca^{2+}]_i$.

This assay was performed at two excitation wavelength, 340 for evaluation of Fura 2-Ca^{2+} complex and 380 for evaluation of free (unbound) Fura 2. Immediately after addition of Cl-IBMECA, florescence emission of Fura 2 was robustly increased at 340 nm, while simultaneously decreased at 380 nm (Fig. 3a), indicating Ca^{2+} is elevated inside the cells. We calculated 340/380 ratio to merge florescence oscillations in these wavelength and indicate elevation of $[Ca^{2+}]_i$ inside MIN6 cells (Fig 3b). Altogether, our results showed that activation of A3AR in MIN6 cells can elevate $[Ca^{2+}]_i$ levels. Apart from cAMP dependent signaling pathways, A3AR stimulation also trigger Ca^{2+} pathways in MIN6 cells.

A3AR stimulation potently increases insulin release from MIN6 cells

MIN6 cells secret insulin in response to different stimuli. Thus, to evaluate effects of Cl-IBMECA on GSIS, we stimulated MIN6 cells with glucose in the presence of this agonist. As presented in Fig. 4, Cl-IBMECA potentiated GSIS at lower concentrations compared with untreated cells ($P < 0.05$). The stimulatory effects of Cl-IBMECA on GSIS were decreased in a dose dependent manner. We used MRS 1220 antagonist to confirm effects of A3AR activation on GSIS. Cells were treated with MRS 1220 (1 μM) plus Cl-IBMECA (0.01-0.1 μM) for 1 h. Our results indicated that MRS 1220 inhibited Cl-IBMECA effects on insulin secretion, indicating specific involvement of A3AR in insulin secretion. This assay indicated that A3AR stimulation possibly potentiated GSIS from MIN6 cells.

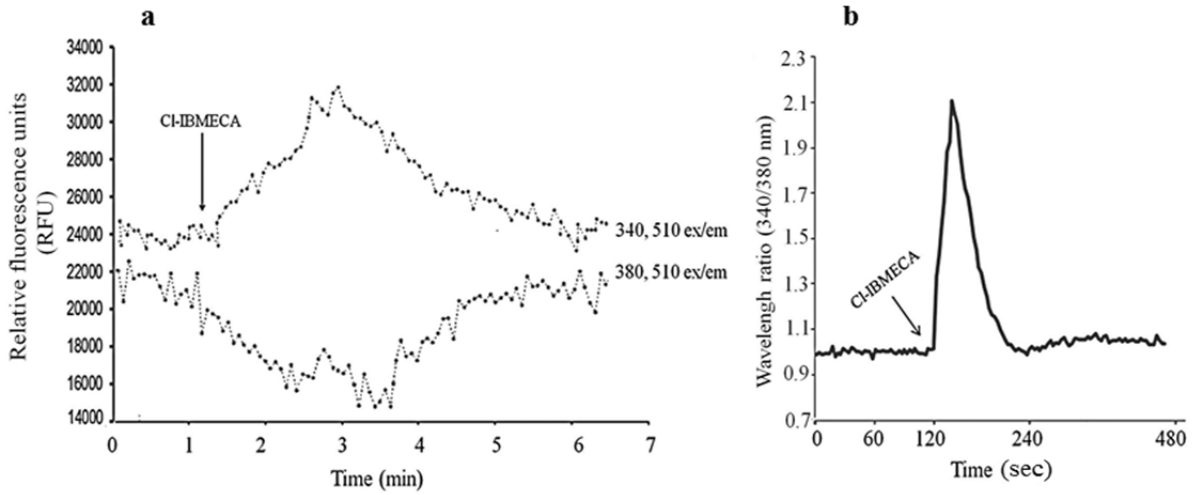

Fig. 3. A3AR mediated elevation of intracellular Ca^{2+} in MIN6 cells. Fura 2/AM (5 μM) was loaded to cells for 1 h and then these cells were stimulated with Cl-IBMECA (1 μM) for 8 min. (a) Fura 2 was stimulated at 340/510 and 380/510 nm to distinguish between Ca^{2+} bound and free forms of Fura 2, respectively. (b) 340/380 Ratio has been evaluated to indicate elevation of intracellular Ca^{2+} following Cl-IBMECA treatment. These assays were triplicated in three independent evaluations (n = 9) and results presented as mean ± SD. A3AR, Adenosine receptor; Cl-IBMECA, A3AR agonist.

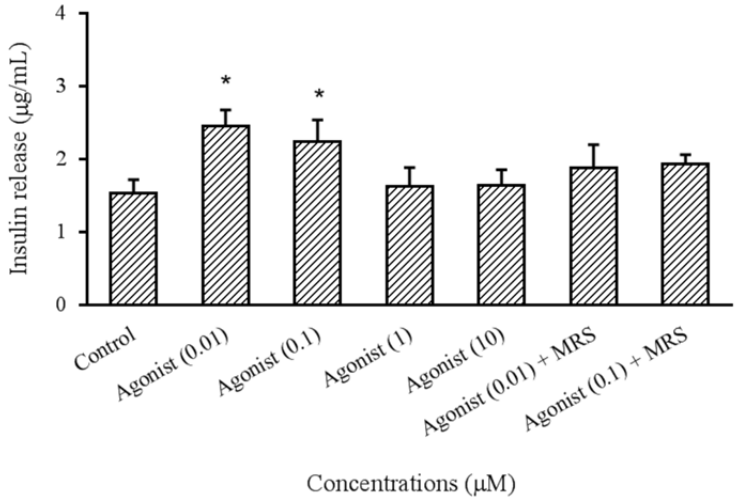

Fig. 4. Cl-IBMECA potentiates GSIS in MIN6 cells. GSIS induced by elevation of glucose levels (from 2.5 mM to 16.5 mM) and then effects of agonists on insulin secretion have been evaluated. MRS 1220 was used as an A3AR antagonist and pretreated at 1µM prior to adding Cl-IBMECA. Insulin content of each treatment was measured using ELISA. This experiment was duplicated in three independent repeats (n = 6) and results were presented as mean ± SD. **P* value < 0.05 in comparison with control group. AR, Adenosine receptor; Cl-IBMECA, A3AR agonist; GSIS, glucose stimulated insulin secretion.

DISCUSSION

Since A3AR signaling mediators were not determined in PBCs, we promoted to determine these mediators in MIN6 insulinoma cells. Our results suggested that MIN6 cells express A3AR of these receptors, however, it's level was lower than A1AR. Chia *et al.* did not found any A3AR expression in mouse isolated islets (8). However, the expression of these receptors was confirmed by others in βTC6 and INS-1 cell lines and even in mouse isolated islets (2, 6).

Classical A3AR signaling pathways are mediated through activation of Gi/o and Gq. These G-proteins lead to decrease cAMP levels and elevation of intracellular Ca^{2+} (17). However, depending on cell type, A3AR may recruit different signaling pathways, which leadto various cellular responses (15). For example, it has been shown that in renal epithelia cells A3AR is coupled to Gs proteins and increase cAMP levels in response to Cl-IBMECA (21). However, A3AR generally considered as a Gi/o coupled receptor which decreases cAMP levels (20). Herein, we also showed, in MIN6 cells that cAMP levels decreased following A3AR stimulation, indicating possible coupling of this receptor with Gi/o proteins (Fig. 5). Previous studies indicated cAMP plays a critical role in insulin secretion and proliferation of PBCs (22). In PBCs, elevated levels of cAMP promotes cell survival and proliferation through insulin receptor substrate 2 (IRS2) and cAMP response element-binding protein (CREB) (23). It has been mentioned that glucose controls insulin secretion by cAMP-dependent activation of protein kinase A (PKA) and Epac signaling pathways (24). Our results indicated that A3AR agonist efficiently (EC_{50} = 5.61 µM) decreased forskolin-induced cAMP levels (Fig. 1b). However, stimulation of this receptor potentiated GSIS from MIN6 cells. It is possible that this receptor engage cAMP-independent pathways to increase insulin secretion (Fig. 5). These pathways may be triggered by increase in $[Ca^{2+}]_i$ by A3AR agonist (Fig. 1c). It has been shown that chelation of $[Ca^{2+}]_i$ (by BAPTA/AM) blockade potentiation of GSIS by glucagon-like peptide-1 (GLP-1) in MIN6 cells (25). At least in cardiomyocytes it is shown that A3AR deactivates ATP-sensitive potassium (K_{ATP}) channels and leads to protection of these cells against ischemic conditions (17). This cannel directly involved in insulin secretion from PBCs (14). Thus it is not possible, in MIN6 cells, activation of this cannel also resulted in potentiation of GSIS, further studies needed.

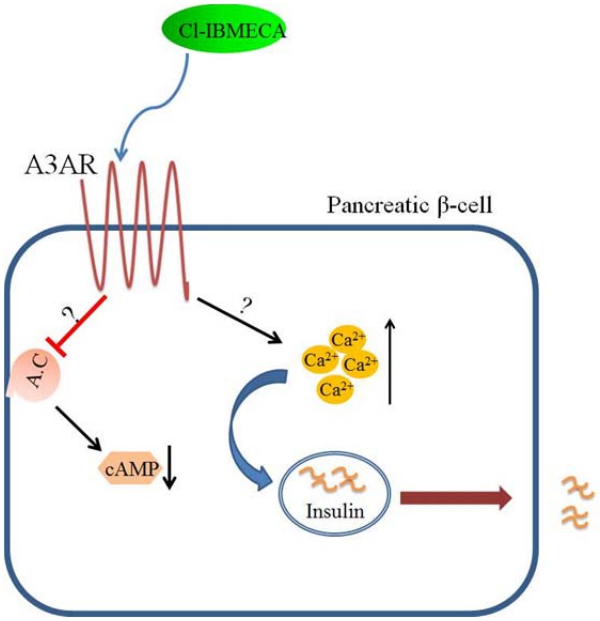

Fig. 5. Possible mechanisms involved in potentiation of GSIS by A3AR. This receptor possibly through elevation of intracellular Ca^{2+} potentiates GSIS. This effect was observed at low concentrations of Cl-IBMECA (> 1 µM). It is possible that at higher concentrations (< 10 µM) cAMP levels decrease extensively and limit potentiation of GSIS. GSIS, Glucose-stimulated insulin secretion; A3AR, A3 adenosine receptor ; Cl-IBMECA, A3AR agonist; AC, adenylyl cyclase.

In vivo studies indicated that Cl-IBMECA mildly increases plasma insulin levels and consequently decreases amount of plasma glucose (26). In parallel, our results also indicated that GSIS was potentiated by Cl-IBMECA. However, these effects in MIN6 cells appear to be concentration dependent, which was stimulatory at concentration between 0.01-1 µM and inhibitory at 1-10 µM. Contradictory, Ohtani *et al.* reported this agonist increases GSIS at higher concentrations (> 10 µM) (6). It is unlikely that at higher concentrations, only A3AR is engaged by this agonist (27). In contrast, Rusing *et al.* reported that Cl-IBMECA *in vitro* and *in vivo*, inhibit insulin secretion from INS-1 pancreatic beta cell line and in GotoKakizaki rats (26). These conflicting results may be related to different experimental conditions.

CONCLUSION

In conclusion, our results indicated that MIN6 cells express active form of A3 subtypes of adenosine receptors. This receptor engages cAMP and Ca^{2+} secondary messengers to propagate signaling in MIN6 cells. A3AR agonist (Cl-IBMECA) at concentrations > 10 µM inhibited GSIS. This effect may be resulted from transient Ca^{2+} entry following stimulation of A3AR. Further studies are needed to reveal application of A3AR agonist (at low concentrations) for potentiation of insulin release from PBCs.

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