

# Metformin Modulates GLP-1- and GIP-Mediated Intracellular Signaling under Normoglycemic Conditions

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## ABSTRACT

GLP-1 and GIP promote insulin secretion from pancreatic  $\beta$ -cells by inducing intracellular signals such as  $\text{Ca}^{2+}$  and cAMP. Metformin primarily acts by inhibiting gluconeogenesis in the liver and promoting glucose metabolism in the muscle. It is used as a concomitant drug with the incretin in the treatment of T2D. We focused on intracellular signals under various glucose concentrations and assessed the effects of metformin on incretin signaling in MIN6  $\beta$ -cells. Metformin inhibited incretin-induced  $[\text{Ca}^{2+}]_i$  in the presence of 5.5 mM glucose but not 16.7 mM glucose. In accordance with low  $[\text{Ca}^{2+}]_i$ , insulin secretion from MIN6 cells declined, despite enhanced incretin-induced cAMP production. Abundant expressions of *Adcy* 6 and 9, which are negatively controlled by  $\text{Ca}^{2+}$  signals, were detected in MIN6 cells. Thus, increasing cAMP production was associated with the inhibition of  $\text{Ca}^{2+}$  mobilization by metformin. However, we show that metformin controls insulin secretion by inhibiting incretin-mediated  $[\text{Ca}^{2+}]_i$  under normoglycemic conditions.

**Keywords:** Metformin; GLP-1; GIP; Calcium; cAMP; Insulin Secretion

## 1. Introduction

Metformin is commonly used in the treatment of type 2 diabetes and improves insulin sensitization and glucose metabolism [1]. Metformin acts mainly by inducing liver and skeletal muscle serine/threonine kinase B1 (LKB1), which activates AMP-activated protein kinase (AMPK) by phosphorylating Thr 172 of the AMPK  $\alpha$  chain [2]. However, in cell free assays, metformin activated LKB1 and AMPK by an indirect mechanism, and failed to induce phosphorylation of AMPK [3]. In another study, over expression of AMPK in MIN6 cells, which is pancreatic mouse  $\beta$  cell lines established from insulinomas, inhibited insulin secretion in the presence of high glucose concentrations [4]. The incretin peptides GLP-1 (glucagon-like peptide 1) and GIP (glucose-dependent insulinotropic polypeptide), which are the gut derived hormones secreted by L cells and K cells, respectively, after

nutrient ingestion, are blood glucose-lowering hormones that potentiate insulin secretion in response to glucose [5]. Receptors of these incretins, GLP-1R and GIPR are G protein-coupled receptors (GPCR) that activate the classical signaling cascade through Gs and adenylate cyclase (*Adcy*), leading to increased cAMP production [6,7]. Incretins can also mobilize stored  $\text{Ca}^{2+}$  in intracellular organelles by activating ryanodine (RYR) [8] and inositol 1, 4, 5-trisphosphate receptors (IP<sub>3</sub>R) [9]. These two small molecules, cAMP and  $\text{Ca}^{2+}$ , are critical for incretin signaling. Reportedly, metformin can inhibit the activities of liver *Adcy* enzymes [10], which include 9 membrane proteins (*Adcys* 1 to 9) and one soluble protein (*Adcy* 10) and produce cAMP from ATP at the plasma membrane. Several signaling molecules, such as  $\text{Ca}^{2+}$ , calmodulin, protein kinase C (PKC), calcineurin (CaN), and G proteins, participate in the regulation of *Adcys*. *Adcys* 5 and 6 are directly inhibited by  $\text{Ca}^{2+}$  [11,12], whereas *Adcys* 1 and 8 are activated by  $\text{Ca}^{2+}$ -CaM bind-

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ing [13,14]. As a regulator of endoplasmic reticulum (ER)  $\text{Ca}^{2+}$  mobilization, cAMP promotes insulin granule exocytosis [9,15]. Protein kinase A (PKA) and cAMP-activated exchange proteins (EPAC), which are cAMP binding signal proteins, play central roles in these mechanisms [15-17]. Hence,  $\text{Ca}^{2+}$  and cAMP can mutually regulate each other under various conditions. In the present study, we examined the responses of these two small molecules to incretins and assessed the actions of metformin against incretin signaling. In addition, we assessed the expression of the key metformin responsive protein LKB1.

## 2. Material and Methods

### 2.1. Maintenance of the MIN6 $\beta$ Cell Line

MIN6 cells were provided by Dr. J. Miyazaki and were cultured in high-glucose (25 mM glucose) DMEM containing 10% fetal bovine serum (FBS), streptomycin, and penicillin according to the provider's procedure [18].

### 2.2. Measurement of cAMP Production

MIN6 cells were transfected with 3  $\mu\text{g}$  of pGloSensor<sup>TM</sup> 20F cAMP plasmid (cAMP sensor protein expression vector; Promega KK., WI, USA) per  $2 \times 10^6$  cells using Neon<sup>TM</sup> Transfection System (Life technologies Co., CA, USA), and  $3 \times 10^5$  transfected cells were seeded into 96 well white plates. Cells were incubated in DMEM containing 0.1% bovine serum albumin (BSA) at 37°C for two days, and the medium was replaced with medium containing indicated concentrations of metformin. After culturing for 2 h, the medium was replaced with  $\text{CO}_2$ -independent equilibration medium (Life Technologies Co, Carlsbad, CA) containing 6% v/v GloSensor<sup>TM</sup> cAMP reagent (Promega) and 0.1% BSA according to the manufacturer's instructions. Cells were incubated at room temperature for a further 2 h and stimulated with 10 nM GLP-1 or GIP in HEPES-buffered saline (HBSS) containing 107 mM NaCl, 6 mM KCl, 1.2 mM  $\text{MgSO}_4$ , 2 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{KH}_2\text{PO}_4$ , 20 mM HEPES, and 5.5 or 16.7 mM glucose at pH 7.4. Luminescence was sequentially measured using a Varioskan Flash (Thermo Fisher Scientific Inc., MA, USA). Data were corrected for basal luminescence and mean  $\pm$  standard deviation (SD) luminescence per well were calculated and presented.

### 2.3. Measurement of Intracellular $\text{Ca}^{2+}$ Concentrations

Two glass coverslips ( $3 \times 10$  mm, thickness 0.12 - 0.17 mm; Matsunami Glass Inc., Osaka, Japan) were set on 24 well plates before seeding and culturing MIN6 cells in DMEM supplemented with 10% FBS in a humidified atmosphere with 5%  $\text{CO}_2$  at 37°C overnight. Medium

was then replaced with DMEM containing 0.1% BSA and indicated concentrations of metformin, and cells were incubated at 37°C for 2 h. Subsequently, the calcium indicator Fura-2 AM (5  $\mu\text{M}$ ) was added and cells were incubated for 40 min in DMEM supplemented with 1% FBS. Cells on coverslips were washed and perfused with HBSS containing 10 nM GLP-1 and GIP. Cytosolic  $\text{Ca}^{2+}$  concentrations were measured using a 2-wavelength excitation method (340 nm/380 nm) according to the previous report [19] and mean background was subtracted from each value. Data are presented as mean  $\pm$  SD peak delta ratios.

### 2.4. Insulin Secretion Analysis

MIN6 cells were seeded into 48 well plates and were cultured to 60% - 70% confluence. Prior to treatment, medium was replaced and supplemented with 0.1% BSA, and cells were incubated at 37°C overnight. Subsequently, the medium was replaced with fresh medium containing 0.1% BSA and indicated concentrations of metformin, and cells were incubated for 2 h at 37°C. Medium was then replaced with HBSS containing 8 mM glucose and 0.1% BSA, and the cells were incubated for 40 min at 37°C. After washing with PBS, cells were incubated in 2 mM  $\text{Ca}^{2+}$  buffer containing 5.5 or 16.7 mM glucose, 0.1% BSA, and 10 nM GLP-1 or GIP for 60 min at 37°C. Supernatants were collected for insulin analysis and cells were lysed for total protein determinations. Supernatant insulin concentrations were measured using a Mouse Insulin ELISA KIT (TMB; AKRIN-011T, Shibayagi, Gunma, Japan). Data were expressed relative to total protein concentrations of lysates.

### 2.5. Western Blotting

MIN6 cells were seeded into 12 well plates and were cultured to 60% - 70% confluence. Prior to treatment, the medium was replaced and supplemented with 0.1% BSA, and cells were incubated at 37°C overnight. Medium was then replaced with new medium containing 0.1% BSA, and cells were incubated for 10 min. Subsequently, indicated concentrations of metformin were added and cells were incubated at 37°C for indicated times. Supernatants were removed and treatments were stopped by the addition of 50  $\mu\text{L}$  lysis buffer containing PhosSTOP and Complete (Roche Diagnostics, Mannheim, Germany). Lysates were sonicated and centrifuged for 5 min at 15,000 rpm at 4°C. Protein concentrations were determined using a BCA protein assay (Thermo Fisher Scientific) and 60  $\mu\text{g}$  of protein/lane were separated by SDS-PAGE using e-PAGEL (ATTO Co., Tokyo, Japan) and were electro transferred onto nitrocellulose membranes using a Trans-Blot Turbo Transfer System (Bio-Rad Laboratories Inc., Hercules, CA) for 30 min at 25 V.

Membranes were then probed with the following monoclonal antibodies: phospho-LKB1 (Ser 428) rabbit mAb (C67A3; Cell Signaling Tech., Danvers, MA), LKB1 mouse mAb (Ley 37D; CNIO, Madrid, Spain), and  $\beta$ -actin mouse mAb (AC-15; Abcam plc, Cambridge, UK). Immunoreactive bands were visualized using an HRP-labeled secondary antibody and enhanced chemiluminescence. Protein bands were quantitated by densitometric analysis using the GS-800 instrument (Bio-Rad).

## 2.6. Quantitative PCR

Total RNA was isolated from MIN6 cells using RNAiso Plus reagent (Takara Bio Inc., Shiga, Japan), and 2- $\mu$ g aliquots were converted into cDNA using an Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Life Technologies Co, Carlsbad, CA). Expression of *Adcy 1* (NM\_009622.1), *Adcy 2* (NM\_153534.2), *Adcy 3* (NM\_138305.3), *Adcy 4* (NM\_080435.1), *Adcy 5* (NM\_001012765.4), *Adcy 6* (NM\_007405.2), *Adcy 7* (NM\_001037723.3), *Adcy 8* (NM\_009623.2), *Adcy 9* (NM\_009624.2), and  *$\beta$ -actin* (NM\_007393.3) mRNAs were determined by real-time PCR using an Applied Biosystems TaqMan Fast Universal Master Mix and gene specific TaqMan primer probe sets (*Adcy 1*, Mm01187829\_m1; *Adcy 2*, Mm00467874\_m1; *Adcy 3*, Mm00460371\_m1; *Adcy 4*, Mm00475491; *Adcy 5*, Mm00674122\_m1; *Adcy 6*, Mm 00475772\_m1; *Adcy 7*, Mm00545780\_m1; *Adcy 8*, Mm00507722\_m1; *Adcy 9*, Mm 00507743\_m1; and  *$\beta$ -actin*, 4352933E). Fluorophore-based detection using TaqMan probes forms the basis for real-time monitoring. Realtime PCR was performed using an Applied Biosystems STEP ONE thermal cycler. Data were normalized to the expression of  *$\beta$ -actin*.

## 2.7. Statistics

Statistical comparisons were performed with simple main effect test, or ANOVA with Bonferroni/Games—Howell post-hoc tests, as appropriate. Results are presented as means  $\pm$  SD. All statistical analyses were performed using SPSS for Windows (SPSS Inc., Chicago, Ill., USA).

## 3. Results

### 3.1. Increasing cAMP Production in Response to Metformin-Induced GLP-1 and GIP

MIN6 cells were treated with 10 nM GLP-1 or GIP in the presence of 5.5 or 16.7 mM glucose, and cAMP production was monitored for 40 min (**Figures 1(a)** and **(b)**). Pretreatment with metformin for 2 h significantly enhanced incretin-induced cAMP production by 1.7-fold in the presence of 5.5 mM glucose, but this was not metformin dose-dependent. In contrast, in the presence of 16.7 mM glucose, metformin had no effect on incretin-

induced cAMP production. These data suggest that metformin positively regulates incretin-induced cAMP production in normoglycemic conditions.

### 3.2. Modulation of GLP-1-Mediated $[Ca^{2+}]_i$ by Metformin

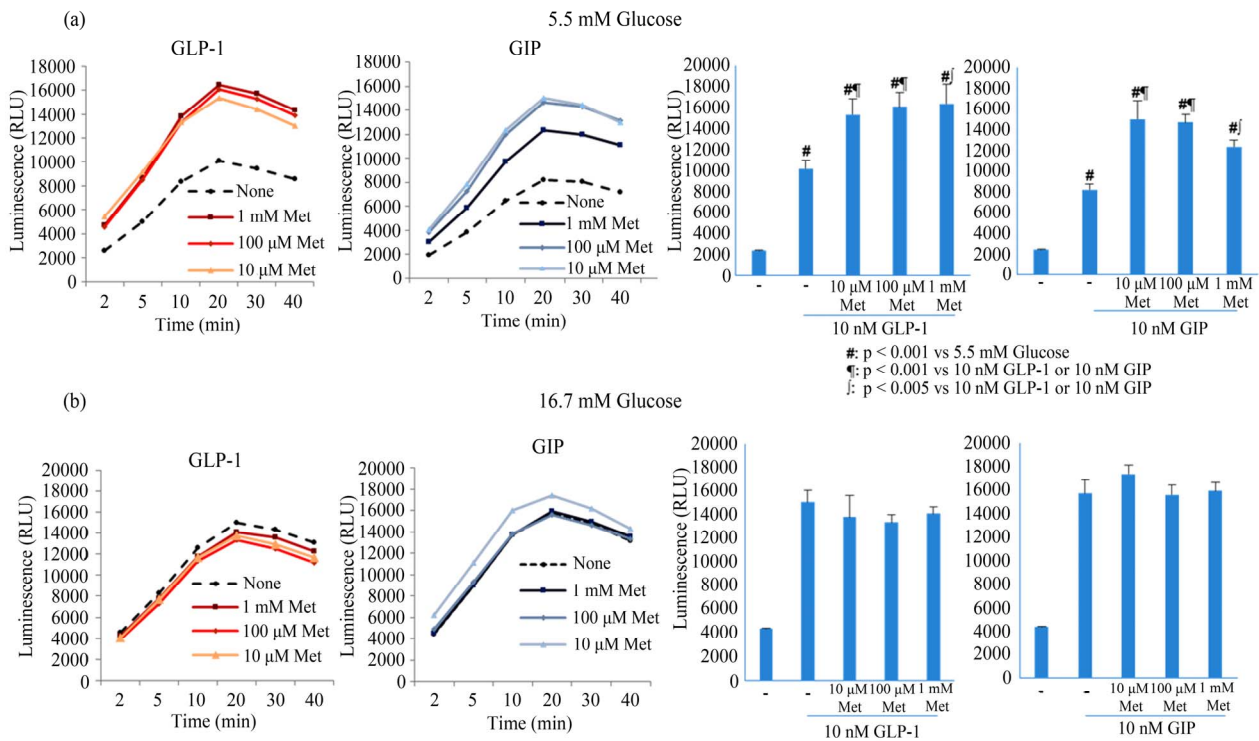
MIN6 cells were stimulated with 10 nM GLP-1 or GIP in the presence of 5.5 or 16.7 mM glucose. Immediate increases in  $[Ca^{2+}]_i$  were observed (**Figure 2**), particularly with GLP-1 under both glucose concentrations, whereas in the absence of added glucose, the response was significantly lower (data not shown). Nonetheless, even in the absence of added glucose, glibenclamide induced a  $Ca^{2+}$  delta ratio response of  $0.423 \pm 0.321$  (diffuse SD value caused by the different oscillation timing of individual cells) by binding the sulfonylurea receptor and activating L-type voltage dependent  $Ca^{2+}$  channels (VDCC; data not shown). Given the small  $Ca^{2+}$  response to GIP, we analyzed the mechanisms of GLP-1-induced  $[Ca^{2+}]_i$ . In the presence of 5.5 mM glucose, GLP-1-mediated increments of  $[Ca^{2+}]_i$  were reduced to approximately 20% after pre-treatment with 1 mM metformin ( $p < 0.05$ , **Figure 2(a)**), but were not decreased in the presence of 16.7 mM glucose (**Figure 2(b)**). Moreover, this decreased depended on pre-incubation time with metformin, with a maximum inhibitory effect after 2 h ( $p < 0.05$ , **Figure 2(c)**). In the presence of 5.5 mM glucose, treatment with 1 mM metformin and 10 nM GLP-1 did not result in decreased  $[Ca^{2+}]_i$  responses (**Figure 2(a)**).

### 3.3. Effects of Metformin on GLP-1- and GIP-Induced Insulin Secretion

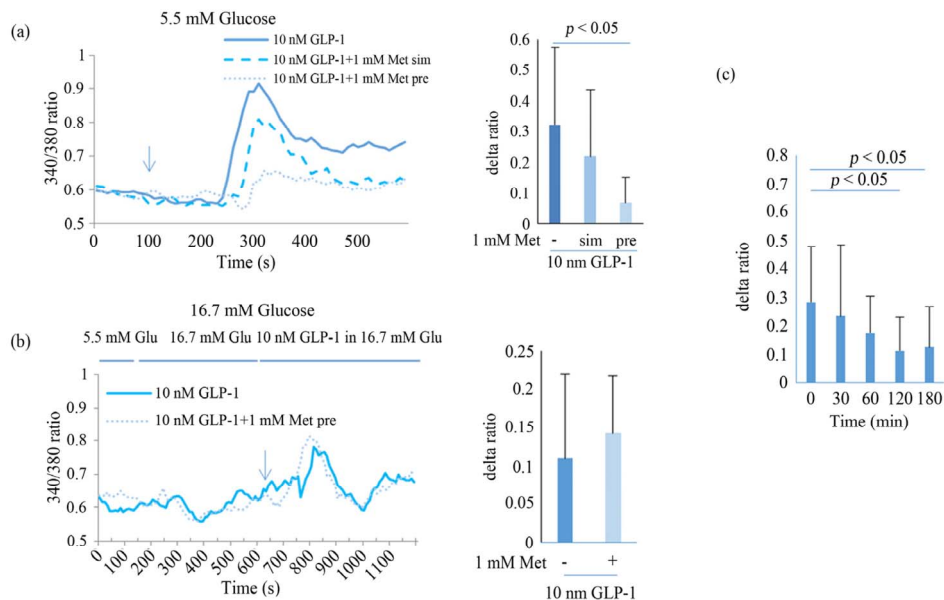
Treatment with 10 nM GLP-1 or GIP enhanced insulin secretion in the presence of 5.5 mM glucose (**Figure 3**), whereas 2 h-pretreatment with  $>100 \mu$ M metformin blocked this effect in the presence of 5.5 mM glucose, no effect of metformin was observed in the presence of 16.7 mM glucose (**Figure 3(a)**). Furthermore, 2 h-pretreatment with  $>10 \mu$ M metformin significantly reduced GIP-induced insulin secretion in the presence of 5.5 mM glucose, but not in the presence of 16.7 mM glucose (**Figure 3(b)**). These data corroborate the inhibition of  $Ca^{2+}$  responses by metformin and suggested that  $Ca^{2+}$  plays a more critical role in insulin secretion than cAMP.

### 3.4. Activation of LKB1 by Metformin

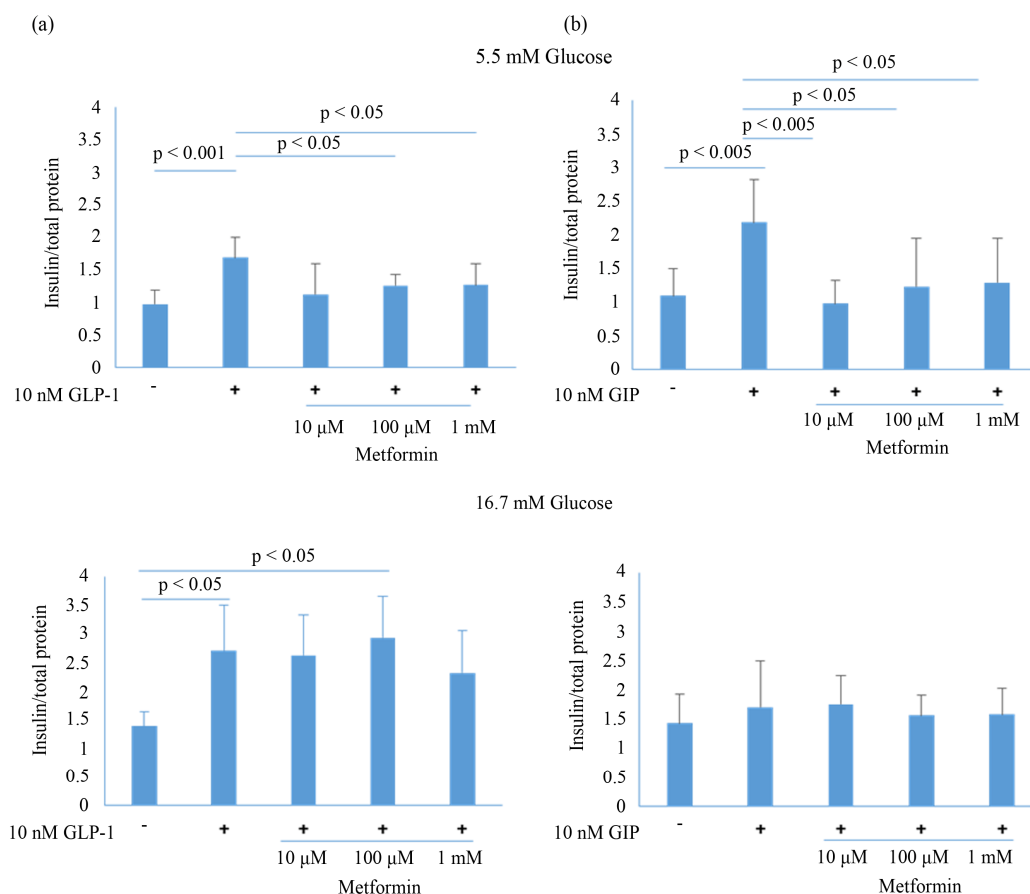
Metformin is known to induce phosphorylation of LKB1 at Ser 428. Thus, we determined LKB1 phosphorylation following treatment with metformin and observed peak LKB1 phosphorylation after treatment with  $>10 \mu$ M metformin for 60 min ( $p < 0.05$ ; **Figures 4(a)** and **(b)**). Vehicle alone (None) induced LKB1 phosphorylation but



**Figure 1.** The influence of metformin on cAMP production in MIN6 cells treated with 10 nM GLP-1 or GIP in the presence of 5.5 (a) or 16.7 mM glucose (b). MIN6 cells were stimulated with 10 nM GLP-1 or GIP in HBSS after 2 h-pretreatments with 0  $\mu$ M (circle), 10  $\mu$ M (triangle), 100  $\mu$ M (rhombus), 100  $\mu$ M (square), and luminescence was measured for 40 min. Luminescence at 20 min is shown in bar graphs. All data were reproduced in 3 - 5 independent experiments. Data are presented as mean  $\pm$  SD and differences were identified using ANOVA with Bonferroni post-hoc test. Met; metformin.



**Figure 2.** Effect of metformin on  $\text{Ca}^{2+}$  responses to 10 nM GLP-1 in MIN6 in the presence of 5.5 (a) or 16.7 mM glucose (b). MIN6 cells were perfused with HBSS containing 10 nM GLP-1, and cytosolic  $\text{Ca}^{2+}$  concentrations were measured using a 2-wavelength excitation method (340/380) for 10 min (solid line of left panel). MIN6 cells were pretreated with 1 mM metformin for 2 h (thin dotted line of left panel) or simultaneously with 10 nM GLP-1 (thick dotted line of left panel). Delta ratios of each peak are presented in bar graphs. The effects of pretreatment with metformin from 30 - 180 min were analyzed (c). Data are presented as mean  $\pm$  SD of 2 - 3 independent experiments and differences between treatment groups were identified using ANOVA with Games-Howell test. Met, metformin; sim, simultaneously; pre, pretreatment; Glu, glucose.



**Figure 3. Effect of metformin on insulin production in MIN6 cells treated with GLP-1 (a) or GIP (b) in the presence of 5.5 or 16.7 mM glucose.** After 2 h-pretreatment with metformin at 0, 10, 100, or 1000  $\mu\text{M}$ , culture medium was changed to HBSS containing 8 mM glucose, and MIN6 cells were incubated for a further 40 min. Medium was then removed and MIN6 cells were stimulated with 10 nM GLP-1 or GIP in HBSS for 60 min. Data are presented as mean  $\pm$  SD of 3 - 4 independent experiments. Differences between treatment groups were identified using ANOVA with Games-Howell (a) or Bonferroni (b) post-hoc tests.

the peak response was at 120 min. It suggested that vehicle lacking nutrition caused ATP production through activating LKB1-AMPK pathway and metformin could enhance the signaling.

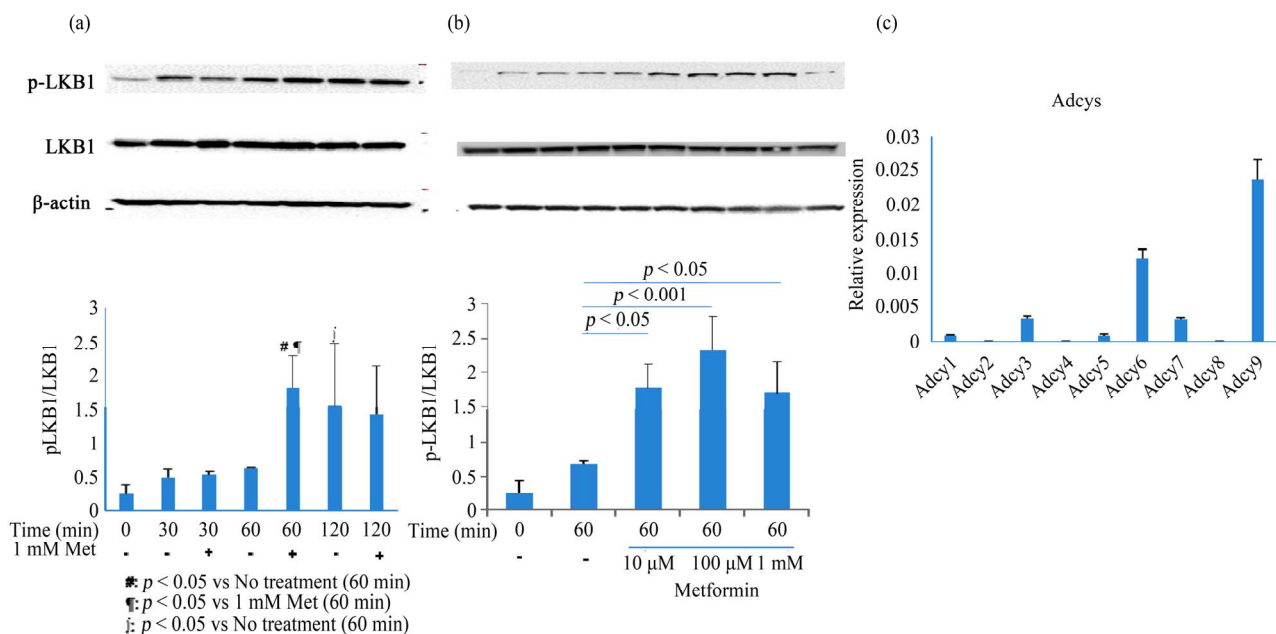
### 3.5. Quantification of Adenylate Cyclase mRNA Expression

Adenylate cyclases synthesize cAMP from ATP; therefore, we analyzed mRNA expression of adenylate cyclases (1 to 9) in MIN6 cells. Real-time PCR data showed that *Adcy* 6 and 9 were expressed at much higher levels in MIN6 cells than the other isoforms (Figure 4(c)).

## 4. Discussion

Metformin is widely used as concurrent treatment for type 2 diabetes mellitus and causes few serious adverse reactions, even at high doses. Its pharmacological actions on glucose metabolism involve the LKB1-AMPK path-

way, which appears to activate ataxia-telangiectasia mutants and activation-induced cytidine deaminase (ATM) [20]. Several other actions of metformin that are independent of glucose metabolism have been reported. Specifically, metformin attenuates neointimal hyperplasia by inhibiting smooth muscle cell proliferation and migration through the insulin-signaling pathway by inhibiting the expression of advanced glycation end product (AGEs) receptors, which are related to cancer mortality through AMPK [21,22]. A recent report demonstrates that administration of metformin increased mRNA expression of the receptors GLP-1R and GIPR in mice. Moreover, *in vitro* metformin treatments enhanced GLP-1R and PPAR- $\alpha$  expression in INS-1 rat  $\beta$ -cells [23]. In this study, GLP-1R expression increased with that of peroxisome proliferator-activated receptor- $\alpha$  (PPAR- $\alpha$ ). However, our *in vitro* analysis in MIN6 cells showed that expression of PPAR- $\alpha$ , GLP-1R, and GIPR were reduced after 24-h treatments with 1-mM metformin in the presence of 5.5 mM glucose (data not shown). MIN6 and



**Figure 4.** Analysis of LKB1 activation and Adcys mRNA expression in metformin-treated MIN6 cells. (a) MIN6 cells were treated with 1 mM metformin for 30, 60, or 120 min. After treatment with metformin, cell lysates (60  $\mu$ g/lane) were electrophoresed and blotted, and protein bands were visualized using chemiluminescence. Typical blots from 2 - 3 independent experiments are presented, and bar graphs show the mean  $\pm$  SD of 2 - 3 independent experiments; (b) Metformin dose-dependency was analyzed at 0, 10, 100, and 1000  $\mu$ M; (c) Adcys 1 - 9 were quantified by real-time PCR using the TaqMan method. Data are presented as the mean  $\pm$  SD of 2 experiments conducted in triplicate. Differences between treatment groups were identified using simple main effect test (a) and ANOVA with Bonferroni post-hoc test (b). Met, metformin; Adcy, adenylate cyclase.

INS-1 cells are commonly cultured in high glucose medium (25 and 11.1 mM, respectively). Differences between INS-1 and MIN6 cells may reflect their respective glucose requirements.

GLP-1 facilitates  $Ca^{2+}$ -induced  $Ca^{2+}$  release (CICR), which is mediated by opening of  $IP_3R$  and RYR cell membrane channels. Holz *et al.* clearly demonstrated that GLP-1-stimulated cAMP was required for activation of CICR by the cAMP binding proteins PKA and EPAC. Moreover, CICR requires continual activation of VDCCs by  $Ca^{2+}$  within intracellular microdomains, but not by robust  $Ca^{2+}$  influx from extracellular spaces [8,15,17,24]. Accordingly, the  $Ca^{2+}$  efflux response to GLP-1 was lower than that induced by glibenclamide, presumably because glibenclamide induces robust  $Ca^{2+}$  influx by opening VDCCs. In the present study, metformin pretreatment inhibited GLP-1-induced CICR and inhibited  $Ca^{2+}$  mobilization and consequent insulin secretion under normoglycemic conditions. However, under hyperglycemic conditions, metformin did not affect either  $Ca^{2+}$  or cAMP responses to incretin signals. In contrast, metformin enhanced cAMP production by GLP-1 and GIP under normoglycemic condition. Although cAMP is generally considered a positive signal for  $Ca^{2+}$  mobilization, the present data indicated that elevated  $[Ca^{2+}]_i$  is indispensable for insulin secretion. Since submicromolar  $Ca^{2+}$

inhibits the functions of Adcys such as Adcy 5, 6, and 9 [25], cAMP production by these Adcys is enhanced when intracellular  $Ca^{2+}$  is depleted. Real-time PCR data showed that the major isoforms expressed in MIN6 cells are Adcy 6 and 9, suggesting that low intracellular  $Ca^{2+}$  levels block inhibition of Adcys. Moreover, metformin enhanced cAMP production and inhibited incretin-induced CICR signaling. Kitaguchi *et al.* showed that extracellular  $Ca^{2+}$  influx-activated Adcy 1 played an important role in insulin secretion following stimulation with 25 mM glucose [26]. High concentrations of glucose activate the  $K_{ATP}$ -VDCC axis by increasing ATP production, and subsequent  $Ca^{2+}$  influx possibly activates Adcy 1. Figure 4(c) shows that Adcys 6 and 9 were the dominant isoforms in MIN6 cells and that Adcy 1 expression was very low. We speculated that incretin-induced cAMP production was dependent on Adcys 6 or 9 under normo-glycemic conditions.

Treatment with metformin activated LKB1 with a peak response at 60 min. Pretreatment with metformin for 2 h also inhibited the  $Ca^{2+}$  response to incretin, with weak inhibition at 60 min. Temporal differences between activation of LKB1 and inhibition of the incretin-induced  $Ca^{2+}$  response were observed after treatments with metformin, suggesting that activation of LKB1 precedes downstream signals. However, the regulatory mecha-

nisms of  $\text{Ca}^{2+}$  mobilization by metformin remain obscure, and further investigations are required to confirm the causal relationship with LKB1. Nonetheless, the present data support mimicry of the physiological function of circulating adiponectin by metformin, which controls incretin-mediated hypoglycemic episodes.

## 5. Conclusion

Metformin has been proven efficacy in the treatment of type 2 diabetes mellitus. Our data indicate a novel function of metformin as a modulator of incretin-mediated insulin secretion. Using *in vitro* analyses, we initially demonstrated incretin-induced insulin secretion under normoglycemic conditions and showed that suppression of this cascade by metformin is blocked in hyperglycemic conditions. We assert that this effect of metformin should be taken into consideration during incretin treatments for the control of blood glucose concentrations.

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## Abbreviations

GLP-1: glucagon-like peptide 1  
 GIP: glucose-dependent insulinotropic polypeptide  
 $[Ca^{2+}]_i$ : intracellular  $Ca^{2+}$  concentrations

Adcy: adenylate cyclase  
 CICR:  $Ca^{2+}$ -induced  $Ca^{2+}$  release  
 T2D: type 2 diabetes mellitus