Published in final edited form as:

Biochem Pharmacol. 2013 April 1; 85(7): 991–998. doi:10.1016/j.bcp.2012.11.029.

AMP-ACTIVATED PROTEIN KINASE AS REGULATOR OF P2Y₆ RECEPTOR-INDUCED INSULIN SECRETION IN MOUSE PANCREATIC β -CELLS

Ramachandran Balasubramanian, Hiroshi Maruoka, P. Suresh Jayasekara, Zhan-Guo Gao, and Kenneth A. Jacobson*

Molecular Recognition Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892

Abstract

5'-AMP-activated protein kinase (AMPK) and its pharmacological modulators have been targeted for treating type 2 diabetes. Extracellular uridine 5'-diphosphate (UDP) activates P2Y₆ receptors (P2Y₆Rs) in pancreatic β -cells to release insulin and reduce apoptosis, which would benefit diabetes. Here, we studied the role of P2Y₆R in activation of AMPK in MIN6 mouse pancreatic β -cells and insulin secretion. Treatment with a potent P2Y₆R dinucleotide agonist MRS2957 (500 nM) activated AMPK, which was blocked by P2Y₆R-selective antagonist MRS2578. Also, MRS2957 induced phosphorylation of acetyl-coenzyme A carboxylase (ACC), a marker of AMPK activity. Calcium chelator BAPTA-AM, calmodulin-dependent protein kinase kinase (CaMKK) inhibitor STO-069 and IP₃ receptor antagonist 2-APB attenuated P2Y₆R-mediated AMPK phosphorylation revealing involvement of intracellular Ca²⁺ pathways. P2Y₆R agonist induced insulin secretion at high glucose, which was reduced by AMPK siRNA. Thus, P2Y₆R has a crucial role in β -cell function, suggesting its potential as a therapeutic target in diabetes.

Keywords

nucleotides; G protein-coupled receptor; insulin; AMPK; diabetes; P2Y₆ receptor

1. Introduction

The global prevalence of diabetes mellitus is rapidly increasing as a result of population aging, urbanization and associated lifestyle changes. Type 2 diabetes is now one of the main threats to human health in the 21st century [1]. It is characterized by hyperglycemia, caused by insulin resistance and/or decreased insulin secretion [2]. The prevalence of type 2 diabetes mellitus is reaching epidemic proportions, and despite an ever-increasing plethora of agents for treating hyperglycemia, only a few drugs (e.g. metformin, pioglitazone) are shown to improve its prognosis [3]. Hence, the identification of new targets that can increase glucose uptake and mitigate the complications of diabetes is required in the search for new type 2 diabetes therapeutics.

*Address correspondence to: Kenneth A. Jacobson, Laboratory of Bioorganic Chemistry, NIDDK, National Institutes of Health, Bethesda, MD 20892-0810, Tel. 301 496-9024, Fax. 301 480-8422, kajacobs@helix.nih.gov.

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5'-AMP-activated protein kinase (AMPK) acts as a sensor of the cellular and whole-body energy status, coordinating catabolism and anabolism [4]. AMPK serves as a key metabolic sensor in both insulin-sensitive and insensitive tissues, because AMPK is capable of responding to changes in intracellular ATP levels. AMPK responds by inhibiting the synthesis of fatty acids and cholesterol [5], inhibiting anabolic pathways by interacting with metabolic enzymes, affecting gene transcription [6] and regulating insulin secretion [7,8]. AMPK, with its important regulatory functions, is considered to be a central mediator in the treatment of type 2 diabetes [9–11].

Various G protein-coupled receptors (GPCRs) expressed in β -islet cells are known to be involved in the regulation of islet function, insulin secretion (especially G_q protein-coupled) and β -cell mass [12]. However, the widespread expression of GPCRs and the complex effects of their activation limit their consideration for diabetes therapeutics. The $P2Y_6$ receptor ($P2Y_6R$), a G_q protein-coupled receptor, is a subtype of the $P2Y$ receptor family for which the native agonist is extracellular UDP. Our laboratory has introduced selective, synthetic ligands for the $P2Y_6R$ [13–15], including the potent agonists 5-iodouridine 5'-diphosphate (MRS2693) and dinucleotide P^1 -(uridine 5'-)- P^4 -(N^4 -methoxycytidine 5'-) triphosphate (MRS2957), and the selective antagonist N,N' -1,4-butanediyl-*bis*[N -(3-isothiocyanatophenyl)thiourea (MRS2578) (Fig. 1). The $P2Y_6R$ activates phospholipase C (PLC) and is involved in increasing intracellular inositol trisphosphate (IP_3) and thus increases intracellular calcium levels. This intracellular calcium acts as a downstream effector for $P2Y_6R$ signaling. The $P2Y_6R$ has been demonstrated to mediate proinflammatory effects, such as stimulation of cell proliferation, recruitment of neutrophils, and release of cytokines in intestinal, endolymphatic, and airway epithelial cells [16]. We recently reported that the activation of the $P2Y_6R$ in MIN6 mouse pancreatic β -cells led to increased insulin secretion and also cytoprotection against apoptosis induced by $TNF-\alpha$ [17]. However, there are no studies detailing the activation of AMPK by $P2Y_6R$.

A number of diverse GPCRs (including adrenoceptors, cannabinoid receptors, ghrelin receptors, and other purinergic receptors) modulate AMPK activity in various tissues. Various G_q -coupled receptors were reported to activate AMPK through their calcium and IP_3 signaling pathways [18,19]. A better understanding of the interactions among GPCRs, AMPK and insulin secretion may help in the development of a potential drug candidate for type 2 diabetes. In this context, we evaluated the mechanisms involved in the activation of AMPK by $P2Y_6R$ agonists and the role of AMPK in $P2Y_6R$ -mediated glucose-stimulated insulin secretion (GSIS) in mouse insulinoma cells.

2. Materials and Methods

2.1. Materials

MRS2578, 2-aminoethoxy diphenylborane (2-APB) and N -[2-[[[3-(4-chlorophenyl)-2-propenyl]methylamino]-methyl]phenyl]- N -(2--hydroxyethyl)-4-methoxy-benzenesulfonamide (KN-93) were purchased from Tocris Bioscience (Ellisville, MO). 7-Oxo-7*H*-benzimidazo[2,1-*a*]benz[de]isoquinoline-3 carboxylic acid acetate (STO-609) and 1,2-*bis*(2-aminophenoxy)ethane- N,N,N,N -tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM) were from Sigma Aldrich (St. Louis, MO). Antibodies to phospho-AMPK, AMPK α 1/2, phospho-acetyl Coenzyme A carboxylase (phospho-ACC), ACC, and anti-mouse and anti-rabbit HRP-conjugated antibodies were purchased from Cell Signaling Technologies (Danvers, MA). $P2Y_6R$ specific antibody was from Alomone Labs Ltd. (Jerusalem, Israel). Goat anti-mouse IRDye 680LT and Goat anti-rabbit IRDye 800 CW were purchased from Li-Cor BioSciences (Lincoln, NE). All other reagents not mentioned were of the highest grade commercially available. MRS2957 was prepared as described with additional synthetic details in the Supporting Information [12].

2.2. Cell culture

A mouse pancreatic β -cell line, MIN6, was used in the present study. MIN6 cells were grown in DMEM with 25 mM glucose in the presence of 15% fetal bovine serum (FBS) under 5% CO₂ and 95% air at 37° C. MIN6 cells between passages 15 and 30 were used for the present study.

2.3. Insulin secretion assay

MIN6 cells were seeded in a 96-well plate at a density of 40,000 cells/well and cultured in DMEM at 37° C. After 48 h, the medium was replaced with Kreb's ringer bicarbonate buffer (KRBB) containing 3.3 mM glucose and 0.3% albumin, for 1 h. Insulin secretion was then induced by supplementing KRBB containing 16.7 mM glucose in the presence or absence of P2Y₆R agonists and/or antagonists/inhibitors in KRBB solution for 1 h at 37° C. Insulin secretion was determined using an insulin ELISA kit with a mouse insulin standard (Crystal Chem, Inc., Downer's Grove, IL).

2.4. Cell treatment and cell lysate preparation

MIN6 cells were cultured in DMEM and washed twice with PBS, and the medium was changed to KRBB containing with 5.5 mM glucose. Whenever inhibitors/antagonists were used, cells were pre-incubated with inhibitor(s) for 15 to 30 min followed by MRS2957 (500 nM) for various time periods. After incubation, cells were placed on ice, washed with ice-cold tris-buffered saline (TBS), suspended in cell lysis buffer (20 mmol/L Tris, pH 7.5, 100 mmol/L NaCl, 1% [vol/vol] Nonidet P40, 1 mmol/L sodium orthovanadate, 100 mmol/L sodium fluoride, 2 μ g/mL aprotinin, 1 μ g/mL leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride), and lysates were centrifuged at 14,000g for 5 min at 4°C [20]. Supernatants were kept at -80°C until used for a Western blot. Protein concentration was measured using a BCA protein assay kit (Thermo Scientific, Rockford, IL).

2.5. Western blot analysis

Cell lysates (30 μ g protein/well) were analyzed under reducing conditions by SDS-PAGE performed according to Laemmli. Proteins were separated on 12% BisTris gel (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membrane by electroblotting. Membranes were blocked according to the manufacturer's instructions and probed with specific antibodies overnight at 4°C. Subsequently, blots were probed with IRdye-conjugated secondary antibody for 1 h and then analyzed using an Odyssey infrared imaging system (LiCor Biosciences, Lincoln, NE). Unless otherwise mentioned in the figure legends, the blots were scanned and images were captured using the Odyssey imaging system. Quantification of the Western blots is explained in detail in the Supporting Information.

2.6. RNA interference studies

MIN6 cells were grown to 60–70% confluency and then were transfected with the desired siRNA using RNAifect transfection reagent (Qiagen, Valencia, CA) following the manufacturer's recommended protocol. The siRNAs against Ca²⁺/calmodulin-dependent kinase kinase β (CaMKK β), AMPK α 1/2 siRNA (mouse), P2Y₆R and control siRNA used for the respective gene knockdown in MIN6 cells were from (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Total RNAs from MIN6 cells were prepared by using a RNA isolation kit from Qiagen. mRNAs were reversely transcribed into cDNAs, and quantified with real-time PCR and normalized to the endogenous GAPDH using ABI Prism 7900HT (Applied Biosystems). The primer sequences used for the gene expression assay were purchased from Santa Cruz Biotechnology Inc., (Santa Cruz, CA). 48 h after transfection MIN6 cells were used for either insulin secretion assay or cell lysate analysis using the procedures described above.

2.7. Statistical analysis

Results are presented as mean $\pm$ SE (n=3). Assays involving treatment with a single drug were analyzed by 1-way ANOVA with Tukey's multiple comparison test, and assays using more than one drug were analyzed using two-way ANOVA with Bonferroni post test. Differences between groups were rated significant at a probability error (P) <0.05. The graphical data were analyzed using the nonlinear curve-fitting program Prism 5.0 (GraphPad, San Diego, CA).

3. Results

3.1. Expression of P2Y₆R in MIN6 cells

The expression of P2Y₆R in MIN6 cells was determined by using a P2Y₆R specific polyclonal antibody, and a P2Y₆R-specific band was visible at the expected molecular weight of ~37 KDa. The result was confirmed by using a P2Y₆R antibody preincubated with the control peptide antigen where the band was not visible (Fig. 2).

3.2. P2Y₆R agonist-induced activation of AMPK

Though MIN6 cells were grown at a high glucose concentration (25 mM) in cell culture, to mimic the physiological conditions the AMPK analyses were performed in KRBB buffer containing 5.5 mM glucose. MIN6 cells were treated with MRS2957 (500 nM) for 2–60 min, and cell lysates were analyzed by Western blots visualized with antibodies to pAMPK- α (Thr-172) and total AMPK- α . A significant increase in the phosphorylation of AMPK- α induced by the P2Y₆R agonist MRS2957 was observed. The increase of AMPK phosphorylation was rapid and reached maximal stimulation (~2–3 fold) 15 min after exposure to MRS2957 (Fig. 3A).

It is well established that phosphorylation of AMPK on Thr172 is associated with AMPK activation [21]. Active AMPK phosphorylates its various downstream targets, including ACC [22]. Western blot analysis of lysates from MIN6 cells treated with MRS2957 showed a significant increase in phosphorylation of ACC (Fig. 3B) when compared to the controls. Thus, activation of AMPK and its downstream signaling are promoted by P2Y₆R activation by agonist MRS2957.

3.3. Phosphorylation of AMPK in MIN6 cells via P2Y₆R activation

A selective, noncompetitive P2Y₆R antagonist MRS2578 was used to confirm that the AMPK phosphorylation induced by MRS2957 was through stimulation of P2Y₆R. Pre-incubation with MRS2578 significantly decreased AMPK phosphorylation when compared to MIN6 cells treated only with MRS2957 (Fig. 4). Thus, the phosphorylation of AMPK induced by MRS2957 is dependent on activation of the P2Y₆R.

3.4. Mechanism of AMPK phosphorylation induced by P2Y₆R agonist

The P2Y₆R increases intracellular calcium concentration by activation of PLC, which mediates many signaling functions of the P2Y₆R. Hence, we investigated the role of Ca²⁺ in P2Y₆R-mediated AMPK phosphorylation. The P2Y₆R acts through G_q proteins to activate PLC and subsequently increase intracellular IP₃ and calcium. To probe the influence of intracellular Ca²⁺, MIN6 cells were incubated with a calcium chelator, BAPTA-AM (10 μ M, 20 min), followed by incubation with MRS2957. BAPTA-AM significantly attenuated P2Y₆R-mediated AMPK activation (Fig. 5A). This suggested that a Ca²⁺ signaling pathway was involved in the phosphorylation of AMPK.

Ca^{2+} can interact with calmodulin to activate CaMKK β , a potential upstream activator of AMPK [23]. STO-609, a specific cell permeable inhibitor of CaMKK (1 $\mu\text{g/mL}$) inhibited MRS2957-induced AMPK activation in MIN6 cells (Fig. 5B). Also, CaMKK β siRNA was used to silence its expression in MIN6 cells. The CaMKK β gene-silenced MIN6 cells showed decreased AMPK phosphorylation in comparison to wild type MIN6 cells when treated with MRS2957 (Fig. 5C). The results confirmed that P2Y $_6$ R-activated AMPK phosphorylation is mediated through the CaMKK β pathway.

Finally, to investigate the role of liver kinase B1 (LKB1, a protein kinase that acts as a primary upstream activator of AMPK) in P2Y $_6$ R-induced phosphorylation of AMPK, we used HeLa cells that are deficient in LKB1 [24]. However, HeLa cells are reported to express an endogenous P2Y $_6$ R [25,26]. HeLa cells stimulated with MRS2957 showed a significant increase in AMPK phosphorylation and also activation of ACC when compared to control cells (Fig. 6A, B). This indicated that MRS2957 was able to induce phosphorylation of AMPK even in the absence of LKB1.

3.5. Effects on insulin secretion

Earlier reports by our group [17] and by Parandeh et al. [27] have shown that UDP, by activating the P2Y $_6$ R, stimulated insulin secretion in MIN6 cells and mouse beta cells respectively. Here, we evaluated the GSIS by the selective P2Y $_6$ R agonist MRS2957 in MIN6 cells. Cells were incubated with 500 nM MRS2957 in the presence of 16.7 mM glucose for 1 h, and insulin released into the medium was determined. MRS2957 significantly increased the GSIS when compared to control cells treated with glucose alone, consistent with the previous findings. Also, pretreatment with P2Y $_6$ R antagonist MRS2578 significantly decreased the insulin secretion induced by MRS2957 (Fig. 7A). We have also determined the effect of varying concentrations of MRS2957 on insulin secretion in the presence of 16.7 mM glucose where MRS2957 (100 nM to 10 μM) significantly increases insulin secretion in MIN6 cells (Supporting Information, Fig. S5). 500 nM MRS2957 was the lowest concentration to achieve the peak effect on insulin secretion. We also compared stimulation of insulin secretion by P2Y $_6$ R activation at different concentrations of glucose (3.3 mM, 5.5 mM and 16.7 mM) in MIN6 cells (Fig 7B). A significant difference between control and MRS2957 treated-MIN6 cells was observed only at high glucose concentrations (16.7 mM).

3.6. Role of AMPK in P2Y $_6$ R-induced insulin secretion

The interaction between AMPK and P2Y $_6$ R in β cell insulin secretion was studied using P2Y $_6$ R siRNA and AMPK α 1/2 siRNA in MIN6 cells. We observed ~70% reduction in the expression of each individual gene and the percentage of knock down of gene expression is in Fig. S3. The AMPK α 1/2 siRNA and P2Y $_6$ R siRNA transfected MIN6 cells were used for the insulin secretion assay. As expected, there was a significant difference in insulin secretion subsequent to MRS2957 treatment between control and P2Y $_6$ R siRNA-treated MIN6 cells. In the case of AMPK siRNA transfection, there was no difference between control and AMPK siRNA cells exposed to glucose alone. In the MRS2957-treated groups, enhanced insulin secretion in AMPK-gene silenced MIN6 cells was significantly lower than in control cells (Fig. 8), suggesting that AMPK is involved in the P2Y $_6$ R-mediated insulin secretion.

The above results clearly demonstrate that P2Y $_6$ R stimulation leads to significant insulin secretion when compared to glucose-alone controls. AMPK may also play a role in this increased insulin secretion.

4. Discussion

AMPK is now widely considered as a therapeutic target for the treatment of type 2 diabetes and metabolic disorder based on its effects on whole body metabolism [9–11,28]. There are many reports that confirm the beneficial influence of AMPK activation, for example by metformin, on glucose and lipid metabolism through GLUT4 translocation in skeletal muscle, and conversely, inhibition of gluconeogenesis in hepatocytes and adipocyte hypertrophy in AMPK-knockout mice [18]. However, there are contradictory reports concerning the relationship of AMPK and GSIS [29].

AMPK can be activated by the allosteric action of AMP and by phosphorylation of the residue Thr-172 on its catalytic subunit by upstream kinases. We established a relationship between AMPK and the P2Y₆R in α -islet cells. A potent and selective P2Y₆R agonist MRS2957 induced phosphorylation and activation of both AMPK and its downstream target ACC. Furthermore, by using a P2Y₆R selective antagonist, we have shown that this activation of AMPK in MIN6 cells was mediated through stimulation of the P2Y₆R. In vascular endothelial cells, AMPK phosphorylation was mediated by extracellular nucleotides acting at P2Y₁, P2Y₂, and/or P2Y₄ receptors, but the P2Y₆R appeared not to be involved and the effects of UDP were not examined [20]. To evaluate the effect of glucose concentration on P2Y₆R mediated AMPK activation we studied AMPK activation at different glucose concentration ranging from 0 mM to 25 mM glucose. Between 5.5 mM glucose and 16.7 mM glucose, there is a significant increase in the phosphorylation of AMPK by P2Y₆R as depicted in Fig. S4.

We explored the mechanism of activation of AMPK induced by the P2Y₆R by examining known activators, including various upstream kinases [31–33]. The P2Y₆R, a G_q-coupled receptor, acts through PLC to increase intracellular calcium concentration. This increased intracellular calcium is involved in various downstream signaling pathways. To elucidate the role of calcium in the present study, we used BAPTA-AM, a calcium chelator. Cells pretreated with BAPTA-AM before activation with MRS2957 showed a significant decrease in AMPK phosphorylation. The reversal of MRS2957-induced AMPK phosphorylation by STO-609 (CaMKK inhibitor [30]), CaMKK β siRNA and BAPTA clearly demonstrated that P2Y₆R acts through its calcium signaling pathway to activate AMPK. We also used 2-aminoethoxydiphenylborane (2-APB), an antagonist of the IP₃ receptor, which at high concentrations (> 50 μ M) inhibits calcium release from the endoplasmic reticulum. 2-APB (100 μ M, 20 min) significantly attenuated AMPK phosphorylation by MRS2957 in MIN6 cells, which also confirms the role of calcium signaling in P2Y₆R-mediated AMPK activation (Fig. S1).

The tumor-suppressor kinase LKB1, as well as CaMKK β , have been identified in several studies as the most important upstream activators of AMPK [20]. Our study in HeLa cells lacking LKB1 demonstrated that MRS2957 induced phosphorylation of AMPK even in the absence of LKB1. Other kinases, including PI3K, PKC, and CaMK II, have also been identified as potential upstream activators of AMPK. An inhibitor of PI3K, LY294002, failed to attenuate the P2Y₆R-induced phosphorylation of AMPK (data not shown). PKC, which activates AMPK, was also considered as a possible mechanistic pathway. However, a potent inhibitor of PKC, bisindolylmaleimide (BIM), had no effect on P2Y₆R-induced phosphorylation of AMPK (Fig. S2). Thus, by using pharmacological inhibitors, we excluded the latter kinases as upstream activators of AMPK in response to P2Y₆R activation and demonstrated that CaMKK β mediates this process. The L-type Ca²⁺ channel blocker nifedipine was shown to act indirectly through LKB1 to activate AMPK [49]. Nifedipine (100 nM) did not alter the level of AMPK phosphorylation by P2Y₆R agonist MRS2957 in

MIN6 cells (data not shown), which was consistent with our observation that the P2Y₆R activated AMPK independently of LKB1 kinase in HeLa cells.

Our previous study demonstrated that P2Y₆R activation by agonists UDP and dinucleotide Up₃U led to increased insulin secretion in MIN6 cells [17]. Here we used a more selective and potent P2Y₆R agonist MRS2957 [15] to demonstrate increased GSIS in MIN6 cells. Also, the P2Y₆R antagonist MRS2578 significantly blocked P2Y₆R agonist-induced insulin stimulation (Fig. 7). Our results agree with other reports that P2Y₆R agonists stimulate insulin secretion [27,34].

To further understand if AMPK is involved in P2Y₆R mediated insulin secretion, we employed silencing of AMPK gene expression in MIN6 cells. Although cells treated with glucose alone did not show any difference, MRS2957-treated cells showed a significant difference in insulin secretion between control and AMPK siRNA MIN6 cells. Insulin secretion in MIN6 cells treated with MRS2957 was similar in both P2Y₆R siRNA and AMPK siRNA groups. Although we do not know the exact mechanism involved between AMPK and P2Y₆R-mediated insulin secretion, AMPK appears to be required for enhancement of insulin secretion by the P2Y₆R. Though it cannot be said that P2Y₆R depends solely on AMPK for insulin secretion, the results prove that AMPK could also be one of the modulating factors of P2Y₆R-mediated insulin secretion apart from glucose. Thus, the P2Y₆R is involved in AMPK- and glucose-mediated insulin secretion in β -cells as depicted in Fig. 9.

The role of AMPK in insulin secretion of β -cells has been studied widely, but the results are still highly controversial. Many studies from different research groups [6, 29, 35–37] have reported that AMPK negatively regulates β -cell insulin secretion in primary rat islets and various β -cell lines such as MIN6 cells, HIT cells, rat insulinoma INS1 cells. AMPK activator AICAR and various amino acids, as insulin secretagogues, were used to show that AMPK activation decreases insulin secretion. This theory was also supported by other studies, which used thiazolidinediones to observe a decrease in insulin secretion and activation of AMPK [29, 38]. In contrast, Gleason et al. [39] reported that activation of AMPK by AICAR failed to suppress GSIS in a study involving three different β -cell model systems including primary islets. Also, glucose and amino acids induced insulin secretion as well as activation of AMPK. A couple more studies also supported this theory and concluded that activation of AMPK by AICAR also increased insulin secretion. Recently, Fu et al. [29] in reviewing the role of AMPK in β -cell insulin secretion have reported that treatment of MIN6 cells with AICAR increased both AMPK phosphorylation and GSIS. The effect of AMPK on GSIS may be influenced by glucose concentrations in the medium during the entire assay, short or long term activation of AMPK, cell culture conditions, etc.

We tried to understand the role of AMPK in P2Y₆R-mediated insulin secretion using AMPK activator AICAR and AMPK inhibitor Compound C. However, in our studies both AICAR and compound C increased insulin secretion at low glucose (3.3 mM) and high glucose (16.7 mM) concentrations (results not shown). Similar to our findings, Langelueddecke et al. [40] showed that under standard cell culture conditions both AICAR and compound C increased insulin secretion through AMPK-independent mechanisms. Hence, we used siRNA studies to understand the role of AMPK in P2Y₆R mediated insulin secretion. Unexpectedly, knockdown of the AMPK gene decreased the ability of P2Y₆R agonist MRS2957 to induce insulin secretion. Although we do not yet completely understand the mechanism involved in this pathway, the results show that AMPK is required for P2Y₆R mediated insulin secretion.

Nevertheless, similar to other GPCRs, P2Y₆R is distributed in many tissues, and hence the feasibility of therapeutic targeting of P2Y₆R in β cells or AMPK-regulating tissues is still

unresolved. In other systems, P2Y₆R activation is associated with proinflammatory and atherosclerotic effects [41–43], activation of osteoclasts [44], and cardiac fibrosis [45], which could contribute to side effects of P2Y₆R agonists.

In summary, our findings indicate that P2Y₆R activation increases calcium signaling pathways in MIN6 cells to increase phosphorylation of AMPK, which then phosphorylates ACC. At high glucose concentrations, P2Y₆R stimulated insulin secretion in β -cells, and this insulin secretion by P2Y₆R is regulated by AMPK through an unknown mechanism. These two actions in response to a P2Y₆R agonist would be beneficial in type 2 diabetic patients to preserve β -cell function and glucose homeostasis. Current treatments for type 2 diabetes include metformin and the glitazone family of ligands, which mediate some of their therapeutic effects by activation of AMPK [46–48]. In this context, selective and stable P2Y₆R agonists show great potential for development as diabetes drugs. Further studies would be required to precisely establish the role of P2Y₆R in insulin secretion from β -cells and as a novel pharmacological drug target for diabetes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the Intramural Research Program of NIDDK, National Institutes of Health, Bethesda, MD, USA. We thank Prof. T. K. Harden (Univ. of North Carolina) for helpful discussions.

Abbreviations

AMPK	AMP-activated protein kinase
BAPTA-AM	1,2- <i>bis</i> (2-aminophenoxy)ethane- <i>N,N,N,N</i> -tetraacetic acid tetrakis(acetoxymethyl ester)
BIM	bisindolylmaleimide I
CaMKK	Ca ²⁺ /calmodulin-dependent protein kinase kinase
DMEM	Dulbecco's modified Eagle's medium
ECL	enhanced chemiluminescence
FBS	fetal bovine serum
GSIS	glucose-stimulated insulin secretion
HRP	horseradish peroxidase
IP₃	inositol trisphosphate
KN-93	<i>N</i> -[2-[[[3-(4-chlorophenyl)-2-propenyl]methylamino]methyl]phenyl]- <i>N</i> -(2-hydroxyethyl)-4-methoxybenzenesulfonamide
KRBB	Kreb's ringer bicarbonate buffer
LKB1	liver kinase B1
MRS2693	5-iodouridine 5'-diphosphate
MRS2957	P ¹ -(uridine 5'-)-P ⁴ -(<i>N</i> ⁴ -methoxycytidine 5'-)tetraphosphate
MRS2578	<i>N,N</i> -1,4-butanediyl- <i>bis</i> [<i>N</i> -(3-isothiocyanatophenyl)thiourea
PI3K	phosphatidylinositol 3-kinase

PKC	protein kinase C
PLC	phospholipase C
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
STO-609	7-oxo-7 <i>H</i> -benzimidazo[2,1- <i>a</i>]benz[de]isoquinoline-3-carboxylic acid acetate
TNF-α	tumor necrosis factor- α
UDP	uridine 5'-diphosphate
Tris	tris(hydroxymethyl) aminomethane

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FIGURE 1.
Structures of P2Y₆R ligands: nucleotide agonists and selective antagonist MRS2578.

FIGURE 2.

Identification of P2Y₆R in MIN6 cells using western blot. Left Panel shows blot probed with P2Y₆R antibody and right panel shows a parallel blot probed with P2Y₆R antibody preincubated with the P2Y₆R control antigen. Arrow indicates P2Y₆R specific band at ~37 KDa (MW ladder not shown). Lane 1, MIN6 cells without any treatment, Lane 2, MIN6 cells treated with MRS2957.

(A)

(B)

FIGURE 3.

Phosphorylation of AMPK and ACC induced by the P2Y₆R in MIN6 cells exposed to MRS2957 (500 nM) for various time intervals. Cell lysates were subjected to NUPAGE-gel electrophoresis followed by Western blot. (A) Western blot of MIN6 cell lysates with antibodies to pAMPK (Thr-172) and total AMPK. Graph shows the relative phosphorylation of AMPK induced by MRS2957 when compared to controls. (B) Western blot of MIN6 cell lysates with pACC and total ACC antibodies and the graph shows the relative phosphorylation of ACC induced by MRS2957 when compared to controls. Values are mean $\pm$ SEM, for n = 3. *, $P < 0.05$ when compared to controls.

FIGURE 4.

Phosphorylation of AMPK induced by the P2Y₆R in MIN6 cells exposed to MRS2957 (500 nM) pretreated in the presence or absence of P2Y₆R antagonist MRS2578 (500 nM, 20 min). Western blot of MIN6 cell lysates with antibodies to pAMPK and total AMPK. The graph shows the relative phosphorylation of AMPK induced by MRS2957 alone when compared to MRS2957+MRS2578 treatment. Values are mean $\pm$ SEM, for n = 3. *, $P < 0.05$, when compared to cells treated with MRS2957+MRS2578.

(A)

(B)

(C)

FIGURE 5.

Stimulation of P2Y₆R leads to release of intracellular calcium, which phosphorylates AMPK through CaMKK β . MRS2957 (500 nM) was used to study the mechanism of AMPK phosphorylation induced by the P2Y₆R in MIN6 cells, in the presence or absence of BAPTA-AM (A), an intracellular calcium chelator; STO-069, specific inhibitor of CaMKK β (B); and CaMKK β siRNA (C). In (B), a HRP-linked secondary antibody was used, and the image was developed by using chemiluminescent substrate (Thermo Scientific, Rockford, IL). Values are mean $\pm$ SEM, for n = 3. *, $P < 0.05$, in cells treated with MRS2957 alone compared to MRS2957+BAPTA-AM, MRS2957+STO-069 and Control. # $P < 0.05$ in CaMKK siRNA-transfected cells when compared to wild type MIN6 cells.

(A)

(B) **FIGURE 6.**

AMPK phosphorylation induced by P2Y₆R in the absence of LKB1, a major modulator of AMPK activity. HeLa cells were induced by MRS2957 (500 nM) at various time points, and the cell lysates were used for Western blots. Representative blots were incubated with antibodies to pAMPK (Thr-172) and AMPK (A) and pACC and ACC (B). *, $P < 0.05$, when compared to controls. HRPlinked secondary antibody was used, and the image was developed by using a chemiluminescent substrate (Thermo Scientific, Rockford, IL). Ratios are normalized to control.

(A)

(B)

FIGURE 7.

GSIS in MIN6 cells was studied in the presence of (A) MRS2957 (500 nM) and MRS2578 (500 nM) at 16.7 mM glucose or (B) at different glucose concentrations as indicated in the figure. Values are mean $\pm$ SEM, for $n = 3$. *, $P < 0.05$, when compared to controls; #, $P < 0.05$, when compared to MRS2957.

FIGURE 8.

The role of AMPK in P2Y₆R-induced insulin secretion was evaluated using control MIN6 cells and in MIN6 cells transfected with P2Y₆R siRNA or with AMPK siRNA at 16.7 mM glucose. Values are mean $\pm$ SEM, for $n = 3$. *, $P < 0.05$, when compared to 16.7 mM glucose-treated control MIN6 cells; #, $P < 0.05$, when compared to MRS2957 (500 nM)-treated control MIN6 cells.

FIGURE 9.

The mechanism of P2Y₆R-stimulated insulin secretion and AMPK activation in MIN6 cells. Stimulation of P2Y₆R in MIN6 cells by selective agonist leads to activation of PLC through G_q protein signaling, which cleaves phosphoinositides into diacylglycerol (DAG) and inositol trisphosphate (IP₃). IP₃ increases intracellular calcium by Ca²⁺ release from endoplasmic reticulum, which phosphorylates AMPK through a CaMKKβ signaling mechanism. At high glucose concentrations, P2Y₆R acts as an insulin secretagogue and thus increases insulin secretion from β cells. This insulin secretory mechanism of P2Y₆R may also require AMPK.