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(課程博士関係)

学 位 論 文 の 内 容 要 旨

Gs/Gq signaling switch in β-cells defines incretin effectiveness in diabetes

β細胞のGs/Gqシグナルの変換は糖尿病におけるインクレチン効果を決定する

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Tight regulation of insulin secretion from pancreatic β-cells in response to the metabolic status of the body is necessary to achieve normal glucose homeostasis. The pancreatic β-cells achieve this by responding to various physiological factors including nutrients and hormonal and neuronal inputs. Of these factors, glucose is the primary initiator of insulin secretion. Glucose-induced insulin secretion (GIIS) occurs through a sequence of precisely regulated events in pancreatic β-cells. Transport of glucose into β-cells and enhanced glucose metabolism increase the ATP/ADP ratio, thereby closing the ATP-sensitive K^+ (K_{ATP}) channels. The K_{ATP} channels thus play a crucial role in linking the β-cell's metabolic status to its electrical activity for the release of insulin. The β-cell K_{ATP} channels are composed of the pore-forming subunit Kir6.2 (encoded by *Kcnj11*), and the regulatory subunit SUR1 (sulfonylurea receptor 1, encoded by *Abcc8*).

GIIS is amplified by hormones and neurotransmitters. Many of these function by activation of the trimeric G-proteins Gs and Gq. Among them, the incretins glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), which are released by enteroendocrine L-cells and K-cells following meal ingestion, are critical for preventing postprandial hyperglycemia by amplifying insulin secretion in a glucose concentration-dependent manner. However, GIP-induced insulin secretion (GIPIIS) is lost while GLP-1-induced insulin secretion (GLP-1IIS) is preserved in type 2 diabetes (T2D), accounting for the unique therapeutic utility of GLP-1-based treatments. We therefore generated β-cell-specific Kir6.2 KO mice (β*Kcnj11*$^{-/-}$ mice) to enable clarification of the direct role of the β-cell K_{ATP} channel in insulin secretion and glucose homeostasis. We observed that specific deletion of the Kir6.2 gene in β-cells (βKcnj11-/-) severely impairs glucose and tolerance and glucose-induced insulin secretion (GIIS) in mice. The mice did not also respond to sulfonylurea. Therefore, these results indicated that β-cell K_{ATP} channels are essential for maintaining normal blood glucose

levels, GIIS, and sulfonylurea-induced insulin secretion. We then challenged the mice to response to incretin-based drugs such as DPP-4 inhibitors and GLP-1 receptor agonists (GLP-1RAs), which act by amplifying insulin secretion in patients with T2D to investigated whether incretin-based treatments are effective in β*Kcnj11*$^{-/-}$ mice. The DPP-4 inhibitor sitagliptin restored fasting normoglycemia and glucose tolerance in β*Kcnj11*$^{-/-}$ by increasing plasma levels of active GLP-1 as well as GIP and improved the glucose-induced increase in plasma insulin in β*Kcnj11*$^{-/-}$ mice. To determine the relative contribution of each incretin, we used the GLP-1 receptor (GLP-1R) antagonist exendin-9 to block the GLP-1R-mediated component. Exendin-9 abolished the effects of sitagliptin on glucose tolerance and insulin secretion in β*Kcnj*11$^{-/-}$ mice indicating that the effect of sitagliptin is due to increased GLP-1 levels. GLP-1R agonist liraglutide (50 μg/kg) also improved glucose tolerance as well as fasting blood glucose levels and increased insulin secretion in β*Kcnj11*$^{-/-}$ mice. Intraperitoneal injection perfusing the mice with either GLP-1(100 μg/kg) or GIP revealed impaired response to GIP but conserved response to GLP-1. While GLP-1 amplifies GIIS by mechanisms that involve both Gs and Gq, GIP does so only by the Gs-mediated mechanism. Therefore, we examined the expressions of GIP receptor (*Gipr*), GLP-1 receptor (*Glp1r*), adenylyl cyclases (*Adcys*), and phosphodiestrases (*Pdes*) in β*Kcnj11*$^{-/-}$ islets and control islets, but detected no difference. Also, inhibition of adenylyl cyclase abolished the amplifying effects of both GLP-1 and GIP on GIIS in control islets, while in β*Kcnj11*$^{-/-}$ islets, it abolished the amplifying effect of GIP but only moderately reduced the response to GLP-1, indicating that while the effects of both GLP-1 and GIP are mediated almost exclusively by G_S signaling in the β-cells of control mice, the stimulatory effect by GLP-1 is principally independent of Gs signaling in β*Kcnj11*$^{-/-}$ mice. We therefore explored the possible involvement of Gq signaling in the amplifying effect of GLP-1 on insulin secretion in β*Kcnj11*$^{-/-}$ mice using an inhibitor of Gq, YM-254890. Whereas the amplification of GIIS by carbachol, an acetylcholine analog that signals via Gq was abolished by YM-254890 in both control and β*Kcnj11*$^{-/-}$ mouse islets, that of GIP was unaffected by the inhibitor in both control and β*Kcnj11*$^{-/-}$ islets. Intriguingly, the amplifying effect of GLP-1 was not altered by YM-254890 in control islets, but was abolished in β*Kcnj11*$^{-/-}$ islets, suggesting a switch from Gs to Gq signaling. Basal insulin secretion in β*Kcnj11*$^{-/-}$ islets was also significantly reduced by the compound, raising the possibility that the elevation of basal insulin secretion also reflects the enhanced Gq signaling. Likewise, the insulinotropic effects of palmitate and the GPR40 agonist MK-2305 the actions of which are in part or wholly mediated through Gq signaling, were enhanced in β-cells of β*Kcnj11*$^{-/-}$ mice.

We then generated *Kcnj11* knockout β-cell lines (*Kcnj11*$^{-/-}$βCL1) from parental MIN6-K8 by the CRISPR/Cas9 system to further study second messengers. The successful knockout of functional K_{ATP} channels in *Kcnj11*$^{-/-}$ βCL1 and 2 was confirmed by electrophysiology and sulfonylurea-induced insulin secretion as there was no resting conductance sensitive to tolbutamide or diazoxide. Insulin secretory responses to incretins in *Kcnj11*$^{-/-}$βCL1 were similar to those in the islets of β*Kcnj11*$^{-/-}$ mice. cAMP production by GLP-1 in *Kcnj11*$^{-/-}$ βCL1 was decreased significantly compared to that in parental control indicating that Gs signaling is reduced in K_{ATP} channel-deficient β-cells. We also monitored PKC activity using phosphoserine PKC substrate antibody and found that antibody reactivity to a number of proteins was increased in *Kcnj11*$^{-/-}$βCL1 compared to parental control cells under basal conditions, indicating that the DAG/PKC pathway is enhanced in *Kcnj11*$^{-/-}$βCL1. We also assessed the IP_3/Ca^{2+} pathway and measured inositol monophosphate (IP1) content, a more stable downstream metabolite of IP_3. While IP1 was undetectable in parental control cells under basal conditions or following GLP-1 stimulation, it was markedly increased in *Kcnj11*$^{-/-}$βCL1 at both 2.8 and 11.1 mM glucose, an effect enhanced further by GLP-1 but not by GIP (Figure 3F). These changes were antagonized

by YM-254890. Similarly basal $[Ca^{2+}]_i$ measured at 2.8 mM glucose in *Kcnj11*$^{-/-}$βCL1 was significantly higher than that in parental control. Collectively, these results indicate that the amplifying pathway in insulin secretion is switched from Gs to Gq signaling in K_{ATP} channel-deficient β-cells. Since there were no differences in endogenous Gαs and Gαq protein levels between parental control and *Kcnj11*$^{-/-}$ βCL1 (Supplementary Figure 3K), the signaling switch in *Kcnj11*$^{-/-}$ β cells does not result from altered amount of the G-proteins.

To ascertain that GLP-1 directly enhances coupling of GLP-1 receptor and Gq, we utilized the Gq-specific fluorescence resonance energy transfer (FRET) sensor mNG-Gγ-IRES-Gαq-mTq2 .We expressed this FRET sensor in parental control (MIN6-K8) and *Kcnj11*$^{-/-}$ βCL1 to monitor activation of Gq in response to various stimuli. Carbachol (50 μM), a positive control of activation of Gq, clearly induced changes in the FRET response in both parental control and *Kcnj11*$^{-/-}$ βCL1 which were blocked by simultaneous application of YM. We next compared the responses to GLP-1 (1 nM) in parental control and *Kcnj11*$^{-/-}$ βCL1 cells using mNG-Gγ-IRES-Gαq-mTq2. In *Kcnj11*$^{-/-}$ βCL1 but not in parental control GLP-1 induced a FRET response that was comparable in magnitude to that elicited by carbachol, which was abolished by 100 nM YM-254890. By contrast, GIP (1 nM) did not alter the FRET response, indicating that GLP-1 but not GIP directly activates Gq in K_{ATP} channel-deficient β-cells and that neither of them activates Gq in normal β-cells. Finally, we observed that enhanced Gq signaling and reduced Gs signaling are commonly found in various models of persistently depolarized β-cells, including human and mouse islets exposed to either high glucose or sulfonylurea, or diabetic KK-Ay mouse model.

Based on our findings, we propose the following model to explain differential modulation of insulin secretion by GPCRs. In resting β-cells, insulin secretion is low under basal conditions (low glucose and no activation of GPCRs) because of high K_{ATP} channel activity. Under stimulated state (high glucose, high levels of ligands for GPCRs), K_{ATP} channels are closed and the resulting membrane depolarization causes opening of voltage-dependent Ca^{2+} channels and stimulation of insulin secretion. In this condition, GLP-1 and GIP both activate Gs and increase cAMP production, thereby amplifying insulin secretion through PKA- and Epac2-dependent pathways. In persistently depolarized β-cells (regardless of this being a consequence of genetic, pharmacological or metabolic intervention leading to reduced K_{ATP} channel activity), Gq signalingis enhanced even at basal state. This contributes to further inhibition of the K_{ATP} channels and activation of a depolarizing background conductance, leading to sustained elevation of $[Ca^{2+}]_i$. As a result, basal secretion of insulin is stimulated, which disables the normal metabolic regulation of insulin secretion. In such persistently depolarized β-cells, GLP-1 or other ligands for GqPCRs (such as muscarinic agonist and fatty acids) further enhance Gq signaling and amplify insulin secretion.

Therefore, while Gs is the primary signal for amplification of GIIS in normal β-cells, Gq is the primary signal for the amplification in β-cells persistently depolarized by K_{ATP} channel inactivation such as that found in diabetes. Thus, we demonstrate that a Gs to Gq signaling switch determines the differential efficacies of GLP-1 and GIP in improvement of glucose tolerance as well as the amplification of insulin response to glucose in the diabetic state.

神戸大学大学院医学(系)研究科（博士課程）

論文審査の結果の要旨

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論文題目 Title of Dissertation	β細胞のGs/Gqシグナルの変換は糖尿病におけるインクレチン効果を決定する Gs/Gq signaling switch in β-cells defines incretin effectiveness in diabetes		
審査委員 Examiner	主 査 Chief Examiner 小川 琇 副 査 Vice-examiner 木戸良明 副 査 Vice-examiner 中村俊一		

（要旨は１，０００字～２，０００字程度）

By restoring glucose-regulated insulin secretion GLP-1-based therapies are becoming increasingly important in diabetes care. Normally, the incretins GLP-1 and GIP jointly maintain normal blood glucose levels by stimulation of insulin secretion in pancreatic β-cells. However, the reason why only GLP-1-based drugs are effective in improving insulin secretion after presentation of diabetes has not been resolved.

In this study, the candidate has generated β-cell specific *Kcnj11*$^{-/-}$knock out (β*Kcnj11*$^{-/-}$) mice and investigated the role of this channel on incretin-induced insulin secretion. Whereas insulin content did not differ between control and β*Kcnj11*$^{-/-}$ islets, β*Kcnj11*$^{-/-}$ mice exhibited significantly higher fasting blood glucose levels and severe glucose intolerance, as assessed by both oral and intraperitoneal glucose challenge. Plasma levels of total GLP-1 and GIP before and during oral glucose tolerance test were or tended to be higher in β*Kcnj11*$^{-/-}$ mice than those in control mice. The DPP-4 inhibitor sitagliptin restored fasting normoglycemia and glucose tolerance in β*Kcnj11*$^{-/-}$ mice. Sitagliptin increased plasma levels of active GLP-1 as well as GIP in β*Kcnj11*$^{-/-}$ mice beyond what was observed in_control mice, and improved the glucose-induced increase in plasma insulin. The GLP-1 receptor (GLP-1R) antagonist exendin-9 abolished the effects of sitagliptin on glucose tolerance and insulin secretion in β*Kcnj*11$^{-/-}$ mice, indicating that the effect of sitagliptin is due to increased GLP-1 levels. To directly evaluate the distinct insulinotropic potentials of GLP-1 and GIP in β*Kcnj11*$^{-/-}$ mice, the candidate measured insulin secretion *ex vivo* using perfused pancreas, Although the amplification of 1st phase insulin secretion by GLP-1 was reduced in β*Kcnj11*$^{-/-}$ mice, 2nd phase was significantly enhanced such that the total amount of insulin secretion in response to GLP-1 was similar in control and β*Kcnj11*$^{-/-}$ mice. However, both 1st and 2nd phase insulin secretions in response to GIP were reduced in β*Kcnj11*$^{-/-}$ mice and the area under the curve was reduced by ~75%. These results indicate that glucose intolerance of β*Kcnj11*$^{-/-}$ mice is attributable to the defect in response to GIP, but not to GLP-1. While GLP-1 amplifies GIIS by mechanisms that involve both Gs and Gq, GIP does so only by the Gs-mediated mechanism. The candidate also showed that the adenylyl cyclase inhibitor MDL-12330A abolished the amplifying effects of both GLP-1 and GIP on GIIS in control islets. In *Kcnj11*$^{-/-}$ islets, MDL-12330A abolished the amplifying effect of GIP but only moderately reduced the response to GLP-1, indicating that while the effects of both GLP-1 and GIP are mediated almost exclusively by Gs signaling in the β-cells of control mice, the stimulatory effect by GLP-1 is principally independent of Gs signaling in β*Kcnj11*$^{-/-}$ mice. Whereas the amplification of GIIS by carbachol, an acetylcholine analog that signals via Gq, was abolished by YM-254890 in both control and β*Kcnj11*$^{-/-}$ mouse islets, that of GIP was unaffected by the inhibitor in both control and β*Kcnj11*$^{-/-}$ islets. Intriguingly, the amplifying effect of GLP-1 was not altered by YM-254890 in control islets, but was abolished in β*Kcnj11*$^{-/-}$ islets (Figure 3B), suggesting a switch from Gs to Gq signaling. Moreover, the effect of GLP-1 eliminated by YM-254890 in β*Kcnj11*$^{-/-}$ islets, the increase in basal insulin secretion was also significantly reduced by YM-254890, raising the possibility that the elevation of basal insulin secretion reflects the enhanced Gq signaling as well. The insulinotropic effects of palmitate and the GPR40 agonist MK-2305 (1 μM), the actions of which are in part or wholly mediated through Gq signaling, were also enhanced in β-cells of β*Kcnj11*$^{-/-}$ mice. These results collectively indicate that Gs to Gq signaling switch in Kcnj11$^{-/-}$ β-cells accounts for effectiveness of GLP-1 and Gq-specific agonists and ineffectiveness of GIP in amplification of insulin secretion. The candidate also showed that in other models of persistent depolarization including KKAy mouse and non-diabetic human and mouse β-cells of pancreatic islets chronically treated with high glucose, a Gs/Gq signaling switch in β-cells occurred, indicating the pathophysiological importance of Gs/Gq signaling switch in β-cells.

The candidate, having completed studies on the contribution of Gs/Gq signaling switch in β-cells in the incretin effectiveness in diabetes and having advanced the field of knowledge in the area of the pathogenesis and the treatment of type 2 diabetes mellitus, is hereby recognized as having qualified for the degree of Ph.D.(Medicine).