의학석사 학위논문

Role of K_{ATP} channel endocytosis in glucose-induced depolarization in pancreatic β-cells

췌장 베타세포에서 포도당에 의해 유발되는 탈분극에 있어 ATP 민감성 포타슘 통로 엔도사이토시스의 역할

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서울대학교 대학원

의과학과 의과학전공

지 영 선

A thesis of the Master's Degree

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Feb 2015

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Role of K_{ATP} channel endocytosis in glucose-induced depolarization in pancreatic β-cells

by

Young-Sun Ji

A thesis submitted to the Department of Biomedical Sciences in partial fulfillment of the requirements for the Degree of Master of Science in Medicine at Seoul National University College of Medicine

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Professor _____

ABSTRACT

Background: The ATP-sensitive potassium (K_{ATP}) channel, an inwardly rectifying K^+ channel that consists of pore-forming Kir6.2 and regulatory sulfonylurea receptor 1 (SUR1) subunit, in pancreatic β-cells is regarded as a metabolic sensor that couples blood glucose level to insulin secretion. It is generally accepted that closing of K_{ATP} channels by the increase of intracellular ATP is responsible for membrane depolarization in response to high glucose stimulation to pancreatic β-cells. But there is a growing body of evidence that the whole cell conductance mediated by K_{ATP} channels is determined not only by channel open probability but also by the available channel numbers. So in this study, I asked whether high glucose can induce the reduction of the K_{ATP} channel density in the plasma membrane, and if so, whether this endocytosis mechanism contributes to glucose-induced depolarization of the β-cell membrane potentials.

Methods: INS-1 cells were culture in RPMI media. Resting membrane potentials were recorded by perforated patch recording using nystatin. Changes in subcellular localization of K_{ATP} channels were monitored by immunocytochemistry analysis using anti-Kir6.2 antibody. Activation of phospholipase C was monitored by imaging translocation of fluorescence signals from the plasma membrane to the cytosol in INS-1

cells transfected with GFP-labeled PLCδ-pleckstrin homology domain (PHδ-GFP).

Results: The resting membrane potential of INS-1 cells that were incubated in 0 mM glucose for 2 hr was - 64.4 ± 1.5 mV (n = 12). Application of high glucose (17 mM) induced gradual depolarization followed by action potential firing within about 5 min. Immunocytochemistry analysis showed that Kir6.2 signals were translocated into the intracellular compartment by 17 mM glucose. When endocytosis was inhibited by dynasore, a dynamin inhibitor, or transfecting cells with dominant-negative mutant of dynamin, the high glucose-induced action potential firing was abolished, indicating the significant contribution of endocytosis to the cellular response to high glucose. I discovered that 17 mM glucose activated phospholipase C in INS-1 cells, and that PKC inhibitor suppressed action potential firing and endocytosis of Kir6.2 induced by 17 mM glucose. However, receptor-mediated activation of PKC using carbachol or chemical activation of PKC using PMA did not induce action potential firing or endocytosis of Kir6.2.

Conclusions: High glucose induces endocytosis of K_{ATP} channel into the β-cell cytosol compartment and this phenomenon is crucial for depolarization of β-cell membrane. Involvement of PKC pathway in high glucose-induced endocytosis is suggested.

Key Words: K_{ATP} channels, pancreatic β -cell, Resting Membrane Potential, INS-1 cells, Kir6.2, SUR1, AMPK, glucose deprivation (GD), 17 mM high glucose, endocytosis, depolarization, PKC

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
AP	action potential
ATP	adenosine triphosphate
INS-1	the rat insulinoma cell line
GD	glucose deprivation
GFP	green fluorescence protein
GSIS	glucose stimulated insulin secretion
PH domain	pleckstrin homology domains
K_{ATP}	ATP-sensitive K^+ channel
Kir	inwardly rectifying K^+ channel
mTOR	mammalian target of rapamycin
RMP	resting membrane potensial
PKA	protein kinase-A
PKC	protein kinase-C
PLC	phospholipase C
SUR1	sulfonylurea receptors
17G	17 mM glucose solution

INTRODUCTION

The ATP-sensitive potassium (K_{ATP}) channel, an inwardly rectifying K^+ channel that consists of pore-forming Kir6.2 and regulatory sulfonylurea receptor 1 (SUR1) subunit (Tucker *et al.*, 1997), is involved in the electrical activity and numerous physiological processes including the regulation of insulin secretion and the protection of neuronal and cardiovascular cells during periods of metabolic stress (Huopio *et al.*, 2002; Seino and Miki, 2003; Ashcroft, 2005) A mechanism underlying these roles is their ability to couple the metabolic state (ATP/ADP ratio) of the cell to its membrane potential. This ability is conferred by the unique property of K_{ATP} channels: they are inhibited by ATP and activated by ADP. The role of these channels in the regulation of glucose-stimulated insulin secretion has been the subject of intense research (Hupio *et al.*, 2002; Seino and Miki, 2003; Dunne *et al.*, 2004; Ashcroft, 2005; Haider *et al.*, 2005). A rise in blood glucose increases metabolism of glucose in pancreatic β-cells, leading to an increase of ATP, which triggers a cascade of events: K_{ATP} channels close leading to membrane depolarization, activation of voltage-dependent Ca^{2+} channels and influx of Ca^{2+} into the cytosol (Ashcroft and Rosman, 1989). This Ca^{2+} rise in the β-cell stimulates insulin secretion (Grodsky and Bennett, 1966; Milner and Hales, 1967; Dean and Matthews 1968).

ATP is well known to induce K_{ATP} channel closure by binding to the pore-forming subunit Kir6.2, whereas ADP binding reduces the ATP sensitivity of channel inhibition

to cause an increase in K_{ATP} channel opening (Tucker *et al.,* 1997). This energy-dependent regulation of K_{ATP} channel has been regarded to be sufficient to explain the contribution of K_{ATP} channels to the glucose-dependent regulation of the β-cells membrane potentials (Tarasov *et al.,* 2006). But there is a growing body of evidence that the currents though K_{ATP} channels are determined not only by the open probability of the channels but also by the number of available channels on the plasma membrane. Recently, trafficking of K_{ATP} channels to the plasma membrane was highlighted as another important mechanism for regulating K_{ATP} channel activity. It was shown that a PKC activator facilitated endocytic trafficking of K_{ATP} channels, resulting in decreased K_{ATP} currents (Hu *et al.,* 2003), whereas glucose deprivation state regulates K_{ATP} channel trafficking to the plasma membrane via AMP-activated protein kinase (AMPK) in pancreatic β-cells (Lim *et al.,* 2009). More recently, leptin was shown to activate AMPK signaling in β-cell to promote membrane trafficking of K_{ATP} channels and induce membrane hyperpolarization (Park *et al.,* 2013). They provide strong evidence supporting that the β-cell membrane potential is strongly dependent on phosphorylated AMPK level which is determined not only by glucose concentrations but also by metabolic hormones such as leptin. Taken all together, I can conjecture that not only the modulation of K_{ATP} channel trafficking to the plasma membrane but also the endocytosis may play some pivotal roles in the regulation of β-cell excitability.

Although some of the key molecular are found, the involvement of energy-dependent

signaling mechanisms in the regulation of K_{ATP} channels has not been fully studied. In the mechanism of insulin secretion, as has been noted earlier, it is generally known that the secretion of insulin in response to glucose from the β-cells results from depolarization of the cell membrane by closing K_{ATP} channel with ATP (Boyd 3rd, 1988; Ashcroft and Rorsman, 1989). In this scheme, regulation of the open probability of the K_{ATP} channel by intracellular ATP is only taken into account, but the possibility of the changes in surface channel number by glucose has not been considered. So, what I try to do in this paper is to elucidate whether high glucose can induce the reduction of the K_{ATP} channel density in the plasma membrane, and if so, whether this endocytosis mechanism contributes to glucose-induced depolarization of the β-cell membrane potentials. Additionally, I tried to identify signaling mechanism activated by high glucose to induce endocytosis of K_{ATP} channels.

Materials and Methods

INS-1 cells culture

INS-1 cells, the pancreatic β-cells, were cultured (passage 10-50) in RPMI 1640 medium (Sigma) containing 11.1 mmol/l D-glucose supplemented with 10% heat-inactivated FBS, 10 mmol/l HEPES, 100 unit/ml penicillin, 100 mg/ml streptomycin, 1 mmol/l sodium pyruvate, and 50 μmol/l β-mercaptoethanol at 37˚C in a humidified incubator containing 5% CO. Cells were grown in 12-well plates for electrophysiology and on 12 -mm poly-L-lysine-coated coverslips for immunocytochemistry.

Electrophysiology

Resting membrane potential (RMP) was measured at room temperature using a standard perforated patch clamp technique with an EPC-8 amplifier and Pulse software (version 8.67; Heka Electronik) and analyzed using IGOR software. During recordings, the holding membrane potential was −70 mV and all RMP data was obtained in current clamp mode. Patch electrodes were pulled from borosilicate glass capillaries to make the resistance range between 3 and 5 MΩ when filled with the pipette solution. The internal solution contained the following (in millimolars): 30 KCl, 110 K-aspartate, 2.6 $CaCl_2$, 10 HEPES (pH 7.2 with KOH), 0.5 EGTA, 5 EDTA and 200 μM Nystatin is specially added for perforated patch recordings. The bath solution (Normal Tyrode's solution) was composed of the following (in millimolars): 150 NaCl, 5 KCl, 10 Hepes

(pH 7.4 with NaOH), 0.5 $MgCl_2$, and 1.8 $CaCl_2$. The glucose concentration during incubations and experiments was 0 mM (glucose deprivation) or 17 mM (high glucose condition).

Drug treatment

Dynasore, tolbutamide, carbamoylcholin chloride (carbachol) and rapamycin were purchased from Sigma, and GF-109203X and bisindolylmaleimide V (inactive analogue of GF-109203X) were purchased from Santa Cruz Biotechnology. Phobol-12-myristate-13-acetate (PMA) and compound C were each purchased from Abcam and Calbiochem. And BAPTA-AM was purchased from Invitrogen.

Dynasore, tolbutamide and BAPTA-AM were made up as a 100 mM stock solution, and PMA, compound C and rapamycin were made up as a 20 mM stock solution, and GF-109203X, bisindolylmaleimide V, and carbacol were made up as a 10mM stock solution. All drugs were dissolved in DMSO and stored at -20 ˚C.

25 μM dynasore, 5 μM GF-109203X, 5 μM bisindolylmaleimide V, 100 μM BAPTA-AM, 20 μM PMA and 20 μM rapamycin were treated before and during RMP recordings. 20 μM carbacol, and 20 μM compound C were treated during experiments and 100 μM tolbutamide was finally perfused during recordings. Cells were treated with DMSO (0.1 %) as a control. These drugs were diluted in either RPMI for immunocytochemistry or extracellular solution (NT solution) for patch clamp

recordings. Unless otherwise stated, INS-1 cells were pretreated with these drugs for 30 min prior to or throughout experimentations.

DNA transfection

After plating onto 12mm coverslips at a density of 1.5×10^5 cells/ml, INS-1 cells were transiently transfected with 2 μg of plasmid DNA; DYN1-K44A (a dominant-negative mutant of dynamin-1, which was created by replacing the lysine residue with alanine at the amino acid position 44, cloned into pEGFP-C1), DYN1-WT (wild-type dynamin-1 cloned into pEGFP-C1), and GFP-PLCδ-PH (Pleckstrin homology) domain fusion DNA with Lipofectamine 2000 (Invitrogen) in the ratio DNA: Lipofectamine® 2000 1 : 2.5 (according to the manufacturer's protocol) and further cultured for 48 hours. And GFP was used as indicator for screening.

Immunocytochemistry

For Immunofluorescence experiments, INS-1 cells were fixed with 4% paraformaldehyde in PBS for 15min. and next, for K_{ATP} channel staining, cells were permeabilized with 0.25% Triton X-100 in PBS for 10min, blocked with 2% donkey serum in PBS for 30min at room temperature, and incubated with rabbit anti-Kir6.2 (1 : 50, Santa Cruz Biotechnology) antibodies overnight at 4˚C. As the secondary antibody, donkey anti-rabbit Alexa 488 was used. Finally, confocal images were obtained using a

Fluo View-1000 confocal microscope (Olympus).

Results

High glucose induces membrane depolarization and endocytosis of K_{ATP} channels

According to the previous studies, the surface levels of K_{ATP} channels increase in pancreatic β-cells under fasting conditions in vitro (Lim *et al.*, 2009). In the present study, I have focused on the reduction of surface K_{ATP} channel number by 17 mM high glucose and aimed to elucidate whether this endocytic process contributes to glucose-induced depolarization.

Firstly, resting membrane potential (RMP) was measured in INS-1 cells after glucose deprivation treatment (GD) for 2 h using the perforated patch clamp recording. Under this condition, resting membrane potential (RMP) was mean - 64.4 ± 1.5 mV (n = 12), which is similar to what was reported in the previous study (Park *et al.*, 2012).

When 17 mM glucose solution was perfused, RMP began to depolarize after 1-3 min. Depolarization is preceded gradually and finally reached the threshold of action potential (AP) after 3 - 5 min in 17 mM glucose (Fig. 1A). Action potential threshold was - 38.0 ± 1.2 mV (n = 9) and repetitive AP firing lasted for several minutes of recording period in 13 among 17 cells.

Next immunocytochemistry experiment was performed using specific antibody against Kir6.2 to compare the distribution of K_{ATP} channels in the INS-1 cells (Fig. 1B). In GD-treated cell, a distinctive staining pattern representing the translocation of the K_{ATP} channels toward the cell periphery was observed, which is also similar to what was

reported in the previous study (Lim *et al.*, 2009). In 17 mM glucose solution treatment (5 min, 20 min), however, Kir6.2 was localized mostly to intracellular compartments and uniformly distributed throughout the cytoplasm of cells. Each image was also analyzed by using line scan analysis (along a line drawn across the cell membrane) (Fig. 1B, bottom) and these results were statistically tabulated in Figure 1 B right (GD 49. 64 %, n = 6; 17 mM 5 min, 10.71 %, n = 7).

RMP data (hyperpolarization) and immunocytochemistry data (surface trafficking) were reproduced in GD state following previous studies (Lim *et al.*, 2009; Park *et al.*, 2012) and it was observed that K_{ATP} channels anchored near the plasma membrane in GD condition could be translocated to cytosol compartments by 17 mM high glucose with RMP depolarization.

Dynamin inhibition suppresses high glucose-induced depolarization of INS-1 cell membrane and endocytosis

Next I tested the functional significance of the endocytosis-dependent modulation of K_{ATP} channels. Previous studies have reported that K_{ATP} channel endocytosis is dependent on the large GTPase dynamin (Hu *et al.*, 2003). I first examined if dynamin is involved in 17mM glucose-induced membrane depolarization. After pretreatment with dynasore (inhibitor of the GTPase dynamin, 25 μM) for 30 min at GD state (-65.7 ± 0.9 mV, n = 8), subsequent perfusion with 17mM glucose elicited diminished

membrane depolarization (-57.4 ± 1.6 mV, n = 8) and failed to generate action potential as compared with in the absence of dynasore, which was reversed by tolbutamide (selective K_{ATP} channel blocker, 100 μM) (Fig. 2A and C).

I next conducted RMP recordings after 60 minutes long incubations in dynasore and 17 mM glucose together. It was to inspect solely K_{ATP} channel's closing effect on RMP change only by increase of ATP/ADP ratio, assuming that dynasore completely get rid of the possibility of K_{ATP} channel's endocytosis. And 60 minutes after incubation in dynasore and 17 mM glucose, then I observed that there were still no glucose responses. RMP was a mean value of -53.9 ± 1.9 mV (n = 4) (Fig. 2B).

Previous studies have reported that a form of dynamin-1 containing a lysine to alanine mutation at position 44 in the GTPase domain, DYN1-K44A, showed dominant-negative inhibition of endocytosis (Lee *et al.*, 1999). I also tested whether dynamin affects glucose-induced depolarization using a dominant-negative mutant of dynamin-1 (DYN1-K44A) (Fig. 3). The cells expressing DYN1-K44A showed no response to 17 mM glucose and stably maintained hyperpolarization state (Fig. 3B and C, - 60.1 ± 1.6 mV, n = 5) compared to the cell expressing wild-type dynamin-1, DYN1-WT which showed that the 17 mM glucose response normally occurred and action potential threshold was - 40.0 ± 0.8 mV (n = 4) and repetitive AP firing lasted for several minutes of recording (Fig. 3A and C). Although a slight variation exists, the inhibition effects of dynasore and dynamin-mutant were same results.

I also tested the K_{ATP} channel distribution pattern at the same conditions by immunocytochemistry. Kir6.2 remained still near the cell periphery when cell were pretreated with dynasore or co-pretreated with dynasore and tolbutamide (Fig. 2D). There were no statistical differences in the ratio of surface to total Kir6.2 signal obtained from the line scan data between these two groups. (Fig. 2E, 17 mM + dynasore, 40.62 %, n=4; 17 mM+ dynasore + tolbutamide, 37.49 %, n=5). Compared with Fig. 1B right, although there occured small reduction in the ratio of surface to total Kir6.2, it is fairly clear that dynasore inhibit endocytosis of K_{ATP} channel.

These all results indicated that endocytosis of the K_{ATP} channel by 17 mM glucose was suppressed by dynamin inhibition, and subsequently suppressed RMP depolarization of the cell membrane. It means that endocytosis of the K_{ATP} Channel induced by high glucose is a dynamin-dependent process and is significant in glucose-induced insulin secretion pathway and also gives a good clue that controlling endocytosis of the K_{ATP} channel is essential in cellular energy metabolism.

Effects of rapamycin and compound c on resting membrane potential in INS-1 cells

It is well known that glucose metabolism affects most major signal pathways in pancreatic β-cells (Matschinsky FM, 1990); especially mTOR is participating in many vital signals in molecular pathway and recently known as nutrient sensing functions in

pancreatic β-cells with AMPK (Gleason *et al.*, 2007).

To evaluate the possible association of these proteins in K_{ATP} channel regulation, RMP recordings were conducted with rapamycin (mTOR inhibitor, 20 μM). Rapamycin treatment had no suppression effects in 17 mM glucose (n = 6). AP threshold was - 43.0 ± 1.1 mV and followed by repetitive AP firings (Fig. 4A).

From the beginning experiments, AMPK signaling was very important because AMPK is originally known for the key enzyme regulating energy homeostasis with insulin dynamics (Hardie *et al.*, 2003; Hardie., 2007). Especially GD-induced hyperpolarization state was very meaningful because it has been reported that RMP hyperpolarization by GD is induced by AMPK (AMP-activated protein kinase) activation and when AMPK is activated, K_{ATP} channel is translocated to the cell surface (Lim *et al.*, 2009; Park *et al.*, 2012) and recently it was also known that leptin induced signaling promotes K_{ATP} channel trafficking via AMPK signaling in pancreatic β-cell, suggesting that it is enough to make pancreatic β-cell membrane hyperpolarize only by AMPK activation, regardless of glucose concentration (Park *et al.*, 2012).

So in AMPK activated state (GD), compound C (AMPK inhibition, 20 μM) was perfused to INS-1 cells and conducted RMP recordings to inspect only AMPK inhibition effect on RMP change except ATP/ADP ratio effects (Fig. 4B). Then RMP began to increase slowly and also showed small RMP oscillations. After 10 min, the mean value of RMP was reached up to - 50.9 ± 1.1 mV (n = 5).

PKC is involved in high glucose-induced membrane depolarization and endocytosis

It was reported that PKC activation facilitates endocytic trafficking of K_{ATP} channels, resulting in decreased K_{ATP} currents (Hu K *et al.*, 2003) and also well known that glucose metabolism activates most multiple protein kinases, including protein kinase C (PKC) with various signal pathways in pancreatic β-cells (Tian *et al.*, 1989; Warwar *et al.*, 2006).

To investigate whether PKC mediates 17 mM glucose-induced membrane depolarization, I pretreated cells with GF-109203X (inhibitor of PKC, 5 μM) or its inactive analogue, bisindolylmaleimide V (5 μM) for 5 min prior to perfusion with 17 mM glucose solution. In GF-109203X-pretreated cells, membrane depolarization induced by 17 mM glucose was not observed (Fig. 5 A and D, GF-109203X + 17 mM, -61.8 ± 1.9 mV, n = 6), while bisindolylmaleimide V did not suppress RMP depolarization and repetitive AP generation induced by 17 mM glucose (Fig. 5B and D, bisindolylmaleimide V + 17 mM AP threshold - 42.0 ± 1.0 mV, n = 5). These results indicate that PKC is strongly associated with K_{ATP} channel endocytosis induced by high glucose.

Additionally, it is known that pancreatic islet cells contain at least six PKC isoenzymes, (Knutson *et al.*, 1994) and their roles in glucose-dependent response are

not fully defined yet. Among the PKC isoenzymes, there are calcium- independents isozymes (PKC-delta, -epsilon, and -eta) and calcium-dependents isozymes (PKC-alpha, -beta, and -gamma) (Yang *et al.*, 2003).

So next, I conducted RMP recordings in cells pretreated with BAPTA-AM, cell-permeant calcium chelator (100 μM, 30 min) to find the specific PKC functional groups related with high glucose response. There were no high glucose responses with a mean value of - 60.1 ± 1.9 mV in BAPTA-AM- pretreated cells (Fig. 5C and D, n=4). It suggests that PKC isozymes in membrane depolarization induced by high glucose are modulated by calcium-dependent pathway.

Next I also conducted immunocytochemistry experiments and line scanning analysis (Fig. 5E). Kir6.2 was localized in the inner cell compartment in INS-1 cells exposed in 17 mM glucose in the inactive analogue pretreatment, but still located to the cell surface compartment when cells were exposed in 17 mM glucose in GF-109203X (GF + 17 mM glucose 48.79 %, n = 6; inactive analogue + 17 mM glucose 12.54 %, n = 5) (Fig. 5F).

These findings suggested that PKC is involved in K_{ATP} channel internalization by high glucose response and also suggested that PKC is related in allowing pancreatic beta cell's membrane to depolarize sufficiently with K_{ATP} channel endocytosis in dynamin-dependent way in high glucose exposure condition such as at postprandial glucose level. Finally, these results are well suited to previous study that K_{ATP} channel's

endocytic trafficking is regulated by PKC activation (Hu K *et al*, 2003).

PLC activation is induced by high Glucose

Acetylcholine receptor stimulation by carbamoylcholin chloride (carbachol, cholinergic agonist) activates PLC and produces two second messengers, DAG and inositol (1,4,5) triphosphate (IP3). And PKC is also activated with them (Chen NG *et al.*, 1997; Min *et al.*, 2000). As I have seen, a plausible mechanism for the observed reduction in surface K_{ATP} channel is through PKC activation.

So next, to investigate whether high glucose induces PLC activation in INS-1 cells, I used green fluorescent protein (GFP)-labeled PLCδ pleckstrin homology domain (PHδ-GFP) constructs to visualize PLC activation (Gamper *et al.*, 2004; Horowitz *et al.*, 2005). The PH domain of PLCδ associates with specific phosphoinositides (such as Phosphatidylinositol (3,4,5)- trisphosphate and phosphatidylinositol (4,5)-bisphosphate) at the inner surface of the plasma membrane in resting cells, and when the PLC is stimulated, PIP2 hydrolysis by PLC causes PHδ-GFP to translocate from the plasma membrane to the cytosol causing an intracellular redistribution of fluorescence intensity, because its affinity is approximately 10 times higher for inositol (1,4,5) triphosphate (IP3) than for phosphatidylinositol (4,5)-bisphosphate (PIP2) (Varnai and Balla, 1998).

I perfused 17 mM high glucose to INS-1 cells in glucose deprivation state and performed the experimental analysis by live cell imaging with GFP (Fig. 6 A) and also

conducted same experiments to other INS-1 cells with carbachol (muscarinic receptor agonist, 20 μM) (Fig. 6B).

In the live cell imaging with 17 mM glucose, although the change was not shown dramatically, the surface fluorescence slightly decreased and diffused into cytosol compartment. This intensity change can be verified more clearly by the cytosol scanning analysis (red spot) (Fig. 6A, right). GFP was increased rapidly and slowly passed away (wash out) and in carbachol experiment, I also got almost same results (Fig. 6B, right). It suggests that PLC is sufficiently activated by high glucose in INS-1 cells.

Effects of carbachol and PMA on resting membrane potential in INS-1 cells

Based on previous result that high glucose can induces PLC activation, I next conducted the RMP recordings in carbachol to verify whether carbachol alone induces K_{ATP} channel endocytosis and membrane depolarization in INS-1 cells. I observed that there were no glucose response at all in RMP recordings with carbachol alone (n = 6) (Fig. 7A) but when 17 mM glucose is added, RMP started to increase and finally AP was fired (AP threshold was - 43.0 ± 1.4 mV, n = 4) (Fig. 7B). It means that carbachol alone cannot induce endocytosis of K_{ATP} channels and the inhibition effect of K_{ATP} channel is not directly involved with PLC activation pathway. As a result, carbachol cannot simulate glucose-like response by itself in INS-1 cells.

If so, I need to verify PKC activation alone induce K_{ATP} channel endocytosis and

membrane depolarization in INS-1 cells. So I infused PMA (PKC activator, 20 μM) to INS-1 cells to activate PKC directly (Fig. 7C). RMP slowly increased up to mean value of 52.1 ± 1.4 mV (n = 4), but there was no AP firing. When tolbutamide (selective K_{ATP} channel blocker) was added to directly block K_{ATP} channel, action potential firing was initiated. All these RMP records are arranged in Figure 7D.

I then examined whether PKC activation by carbachol or PMA induces endocytosis of K_{ATP} channels, and found that Kir6.2 was still localized to the cell surface in carbachol and in PMA. Failure of inducing endocytosis by carbchol was well suited to RMP recording data. In contrast,PMA failed to induce endocytosis, but induced a significant depolarization (Fig. 7E). So I speculated that PMA may directly inhibit K_{ATP} channel currents, as was shown in the previous study (Bonev *et al*., 1996).

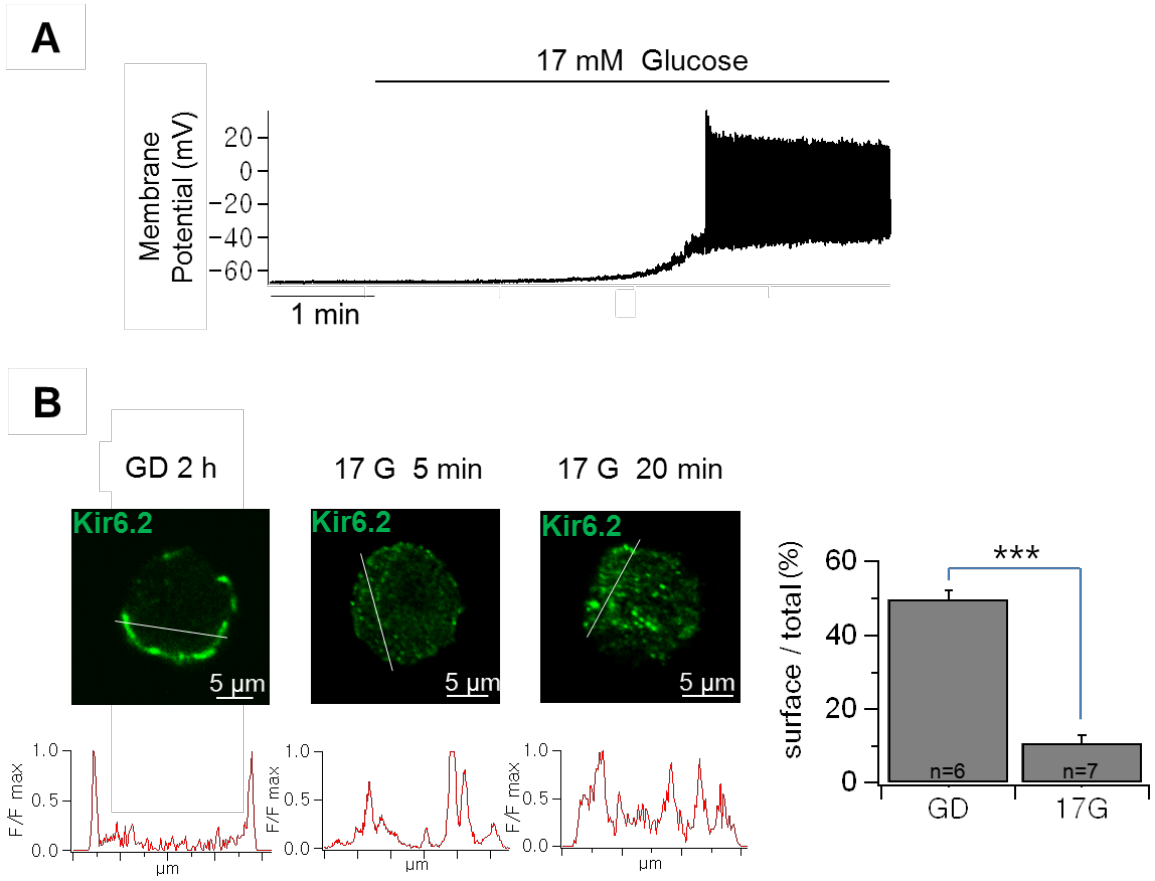

Figure. 1. High glucose induces membrane depolarization and endocytosis of KATP channels. (A) Perforated patch clamp was used to assess resting membrane potentials (RMPs). After 2 h glucose deprivation pretreatment (GD), 17 mM glucose solution was perfused in INS-1 cells (WT) (n = 9) (B) Immunofluorescence analysis using antibody against Kir6.2 in the glucose deprivation state (GD) and perfused in 17 mM glucose for 5 min, 20 min (scale bar = 5 μm). The red graph shows line scanning analysis along a line drawn across the cell membrane. These mean data are analyzed in the bar graph (right). (GD 49. 64 %, n = 6; 17 mM glucose 5 min, 10.71 %, n = 7) ***P < 0.005.

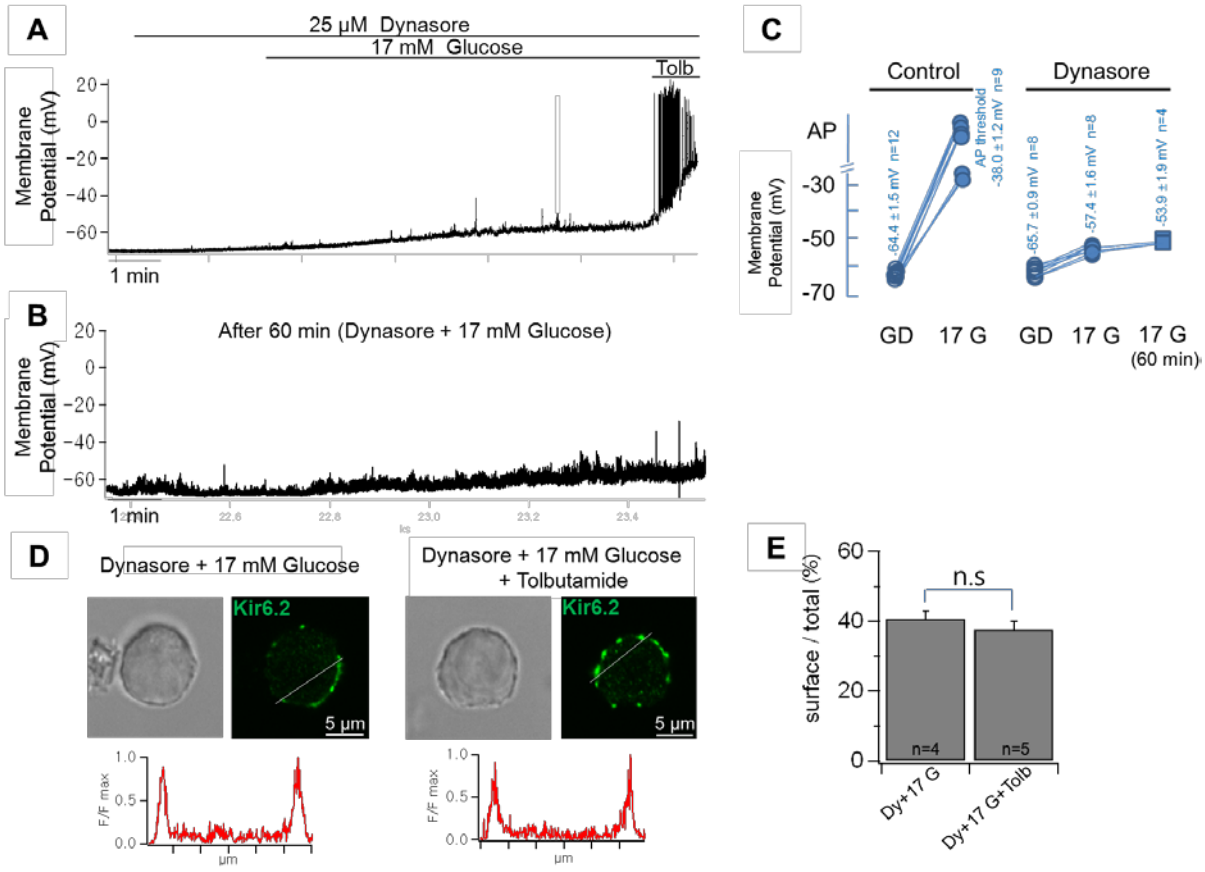

Figure. 2. Dynasore suppresses high glucose-induced depolarization and endocytosis of KATP channels in INS-1 cells. (A) RMP was recorded from INS-1 cells (WT) with dynasore (inhibitor of the GTPase dynamin) pretreatment for 30 min right after glucose deprivation (GD) state and perfused 17 mM glucose. Tolbutamide (selective KATP channel blocker) was finally perfused (n = 8). (B) RMP recordings of 60 min after incubation dynasore with 17 mM glucose (n = 4) and these RMP results are statistically arranged in (C). (D) KATP channel (Kir6.2) distribution pattern in immunocytochemistry at the same conditions in INS-1 cells (scale bar = 5 μm) their mean data (line scan) are analyzed in the bar graph (Fig. 2E, 17 mM glucose + dynasore, 40.62 %, n=4; 17 mM glucose + dynasore + tolbutamide, 37.49 %, n=5) n.s= not significant.

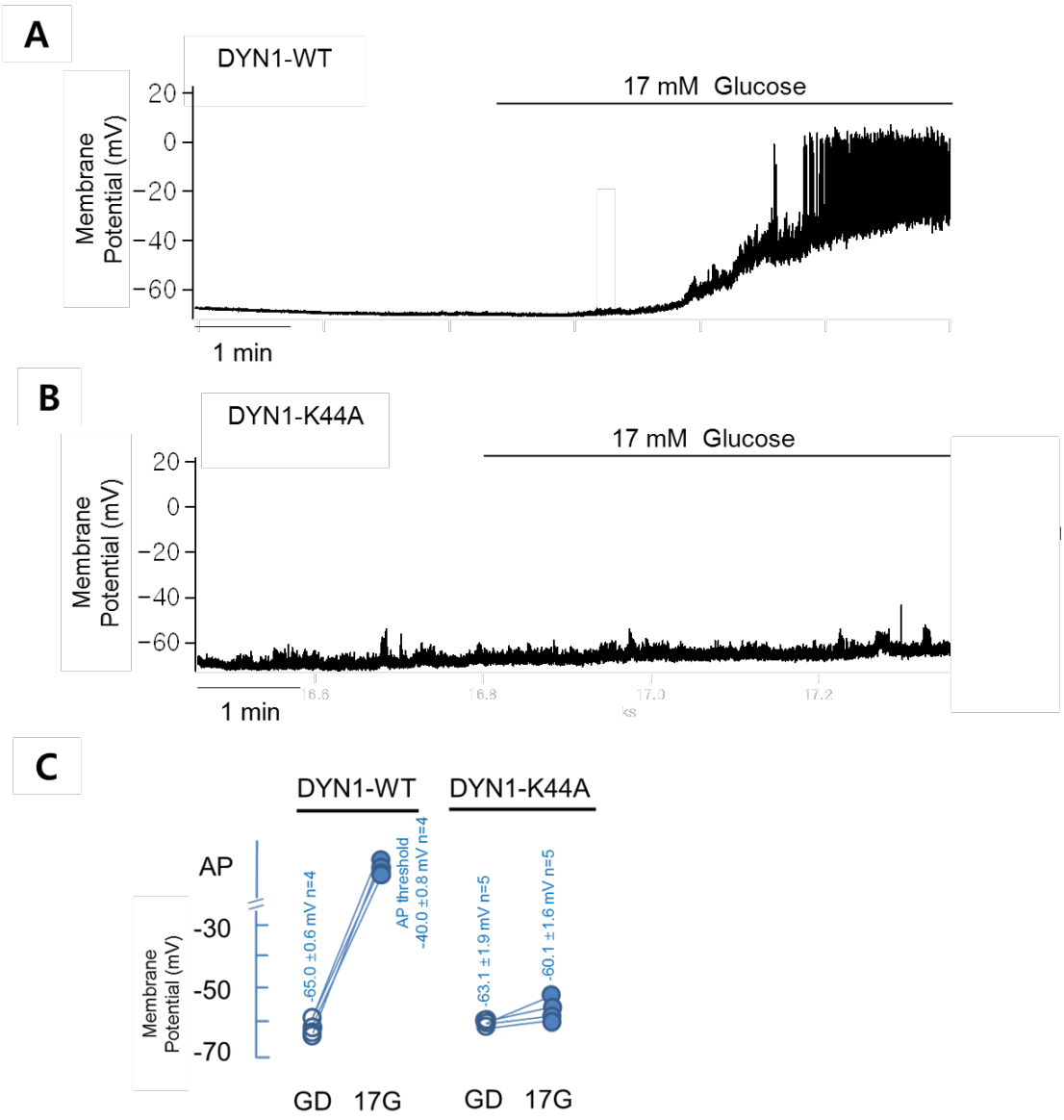

Figure. 3. Dynamin-mutation suppresses high glucose-induced depolarization of INS-1 cells. (A) RMP was recorded from INS-1 cells expressing dynamin1-WT (DYN1-WT) (n = 4). (B) Same recording was repeated from INS-1 cells expressing dynamin1-mutation (DYN1- K44A) in 17 mM glucose solution (n = 5). And these RMP recording data are arranged in (C).

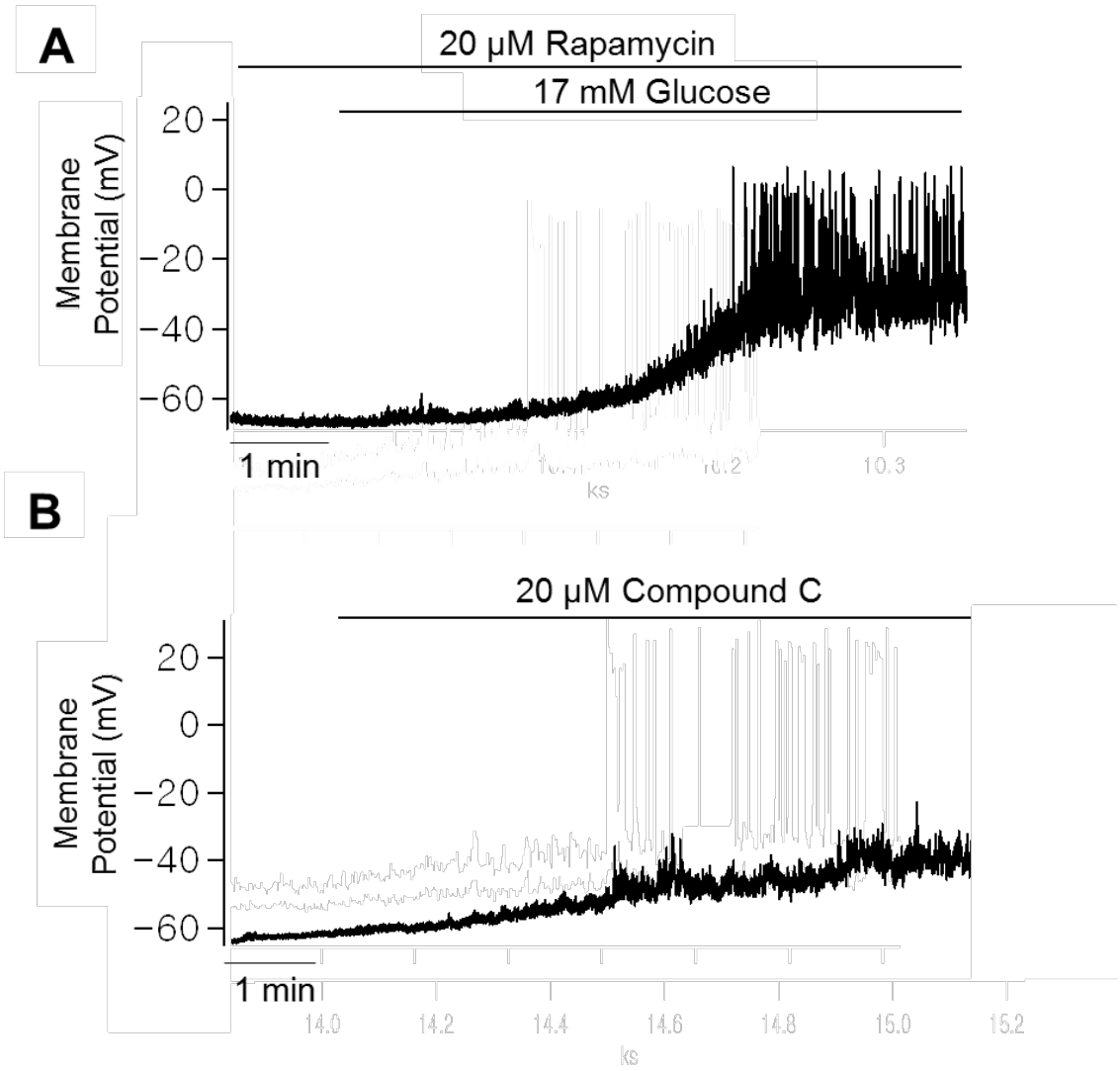

Figure. 4. Effects of rapamycin and compound C on resting membrane potential in INS-1 cells. (A) In glucose deprivation (GD) state, RMP was recorded with 17 mM glucose in rapamycin pretreat (mTOR inhibitor) (n = 6). (B) Compound C (AMPK inhibition) was perfused to see how RMP changes in RMP hyperpolarization state (n = 5).

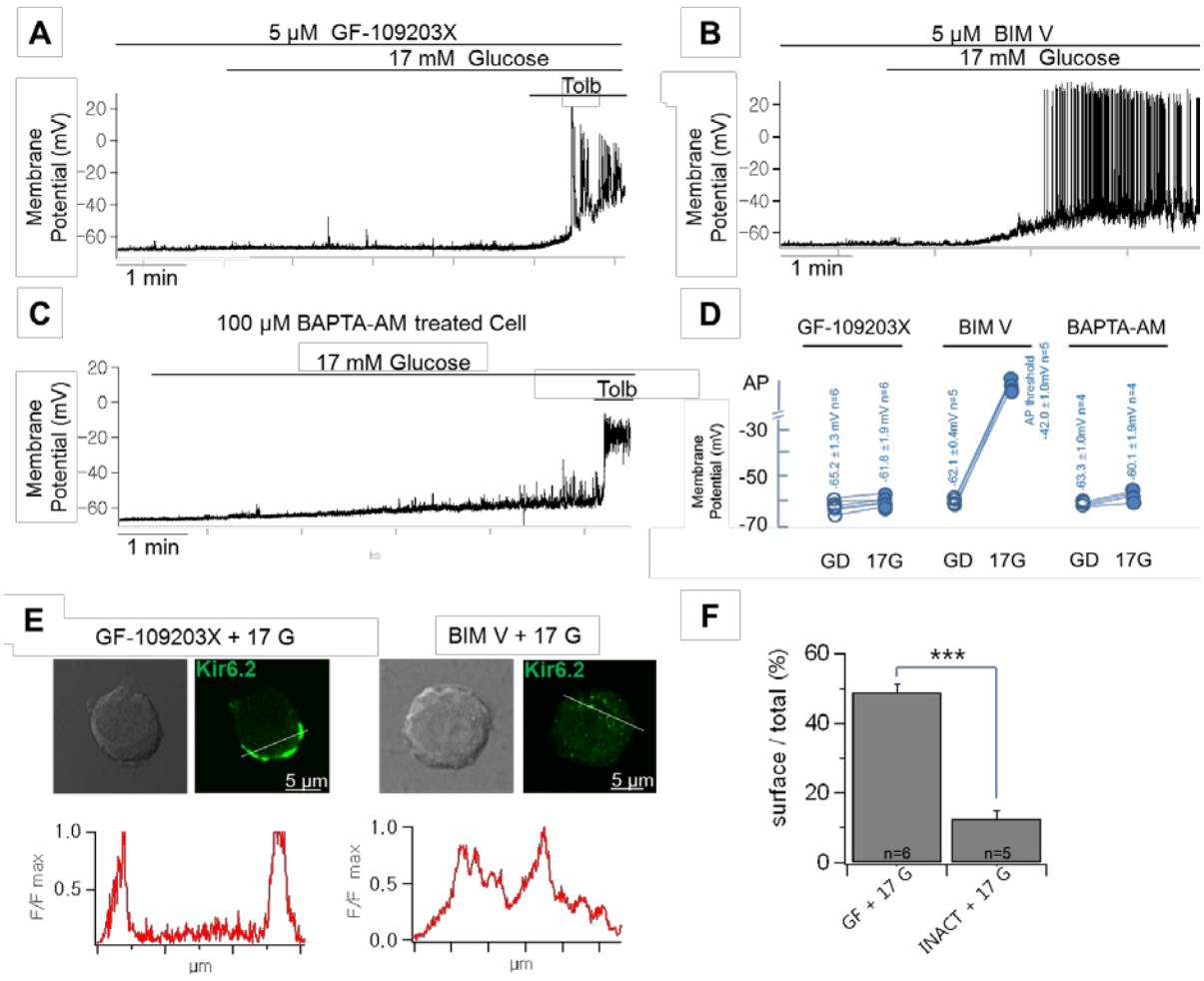

Figure. 5. PKC is involved in high glucose-induced membrane depolarization and endocytosis. (A) GF-109203X (PKC inhibitor) was pretreated for 5 min in glucose deprivation (GD) state, and 17 mM glucose solution was infused to INS-1 cells (n = 7). (B) Same recording was reproduced in Bisindolylmaleimide - V (BIM V) (inactive analogue of GF-109203X) (n = 4). (C) After BAPTA-AM pretreatment (30 min), 17 mM glucose-induced RMP recording was conducted. (E) Immunocytochemistry analysis was also conducted in each condition (scale bar = 5 μm). All results (RMP, line scan analysis) are statistically arranged in (D, F). (GF-109203X + 17 mM glucose 48.79 %, n = 6; BIM V + 17 mM glucose 12.54 %, n = 5) ***P < 0.005.

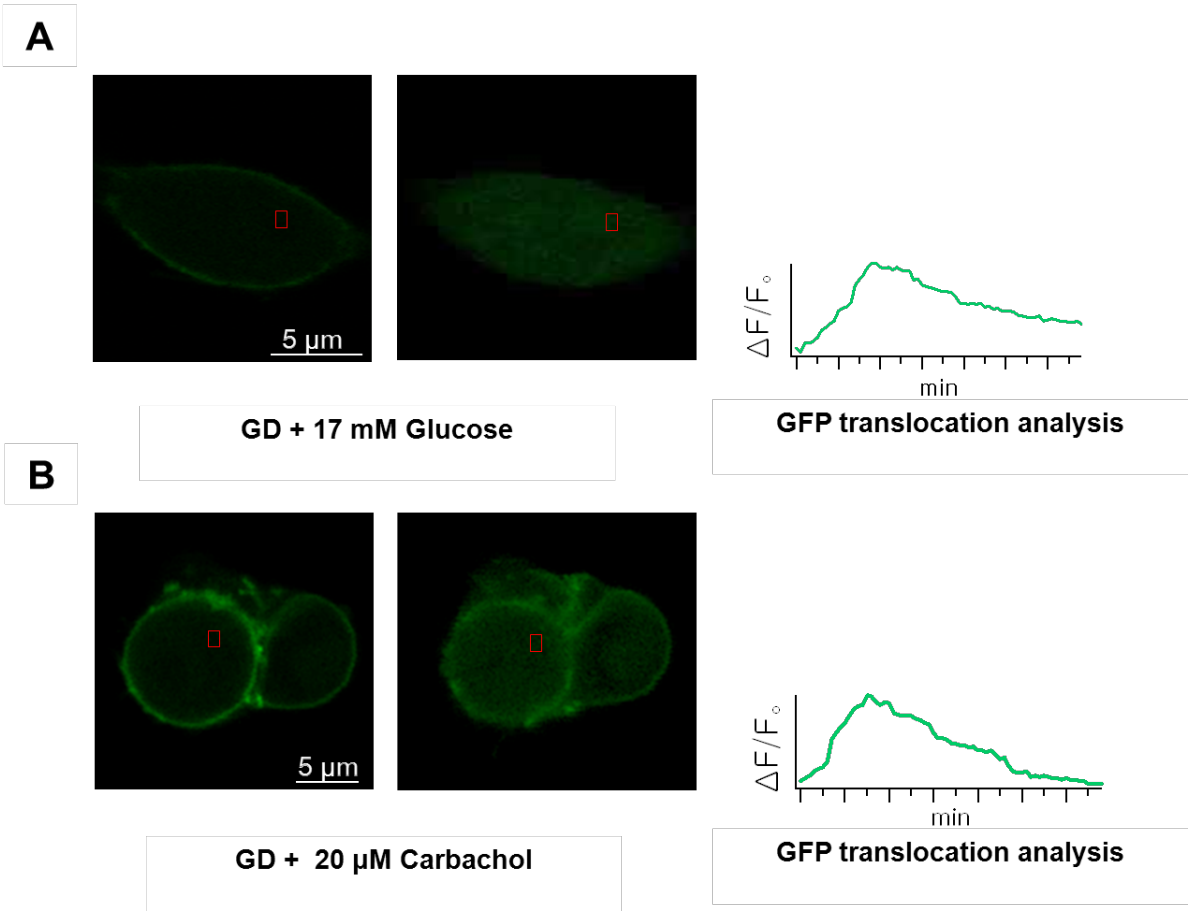

Figure. 6. PLC activation is induced by high glucose. Live cell imaging with GFP variation in GFP-PLCδ-PH domain expression cell. When PLC pathway is stimulated, redistribution of fluorescence intensity was seen in intracellular compartment translocating from the plasma membrane into the cytoplasm by the difference of affinity. (A) The effect of 17 mM high glucose is analyzed in cytosol compartment of INS-1 cell (red spot) through GFP translocation. (B) Same experiments with carbachol (CCh) were conducted and its cytosolic compartment variation is also analyzed in right (green).

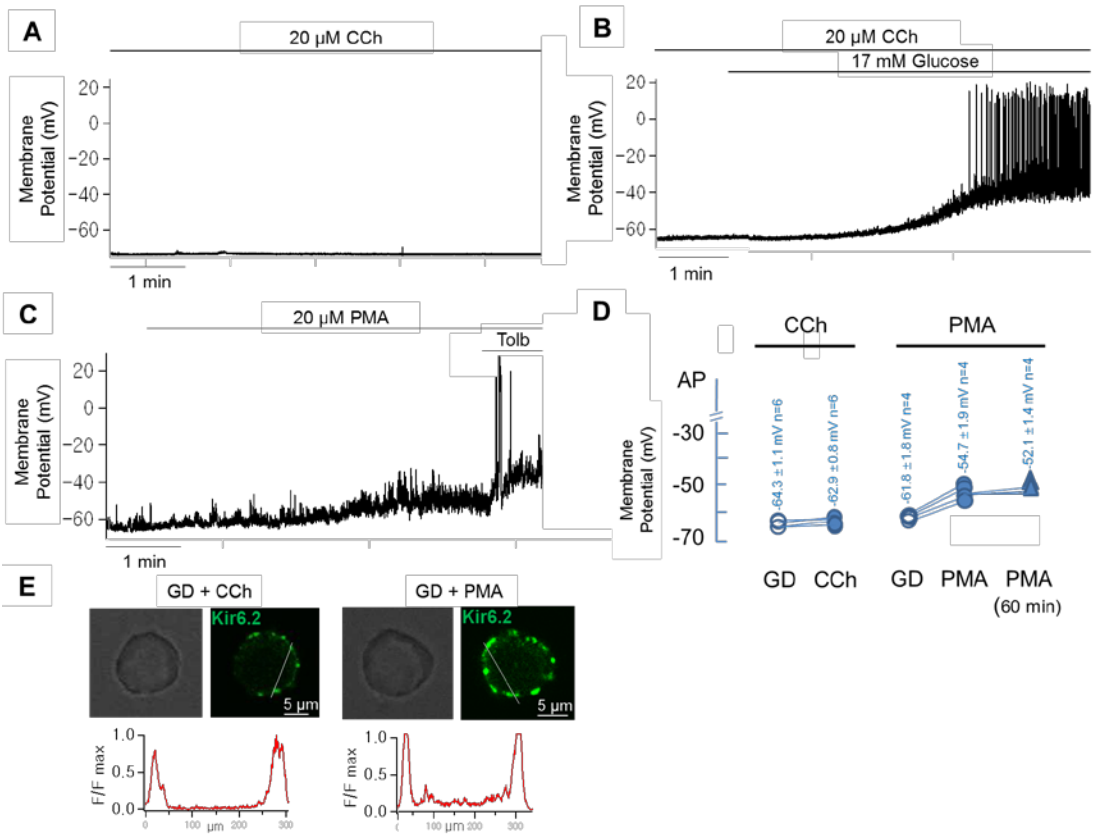

Figure 7. Effects of carbachol and PMA on resting membrane potential in INS-1 cells. (A) RMP recordings in INS-1 cells with carbachol (CCh) alone (n = 6) (B) In carbachol pretreatment, 17 mM glucose was perfused together (n = 3). (C) PMA (PKC activator) and tolbutamide (selective K_{ATP} channel blocker) were used in RMP recordings to reproduce high glucose condition and all RMP results are arranged in (D). (E) Immunocytochemistry image shows K_{ATP} channel distributions in CCh and PMA treatment (5 min).

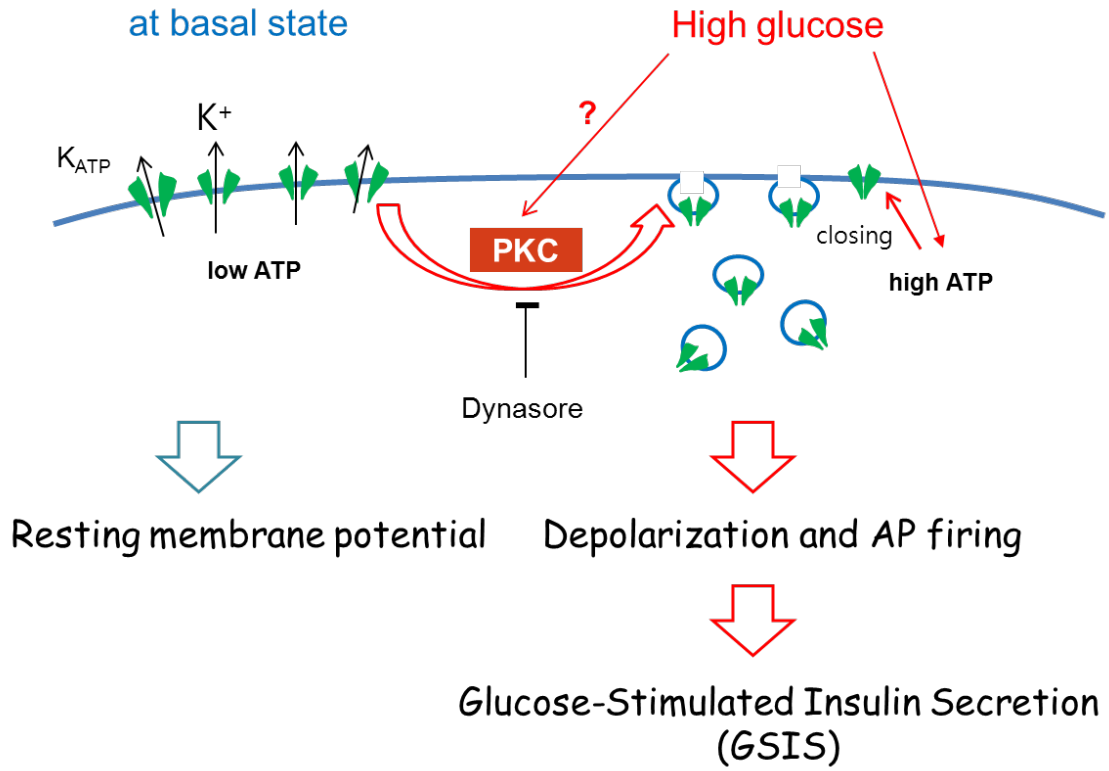

Figure 8. Schematic diagram for the signaling pathway involved in high glucose-induced K_{ATP} channel endocytosis. High glucose-induced K_{ATP} channel endocytosis is regulated in PKC induced down-regulation pathway. K_{ATP} channel regulation by endocytic trafficking and channel closing associates with membrane depolarization and insulin secretion in pancreatic β-cells.

DISCUSSION

The K_{ATP} channel couples blood glucose level to insulin secretion in pancreatic β-cells, which is attributable to its property to be activated at low glucose levels and inhibited at high glucose levels. It has been generally accepted that the change in intracellular ATP concentrations in response to glucose concentrations is responsible for activating or inhibiting K_{ATP} currents (Tarasov *et al.,* 2006). However, recent studies have shown that K_{ATP} channel activation in response to glucose deprivation and leptin shares the same mechanism, which is the activation of AMPK, and that the membrane potential of β-cells is closely related with pAMPK level, rather than glucose level itself (Lim *et al*., 2009: Park *et al*., 2013). Furthermore, they showed that the main action of AMPK is to promote K_{ATP} channel trafficking to the plasma membrane, arguing against the current concept about the mechanism of how K_{ATP} channel acts as a metabolic sensor. They showed that the increase in K_{ATP} channel density is a key mechanism for activating K_{ATP} currents at low glucose levels, but in pancreatic β-cells, the mechanism of K_{ATP} current inhibition at high glucose levels would be more important. Therefore, the first question I asked in this thesis is the contribution of the decrease in channel density by endocytosis to the inhibition of K_{ATP} currents in response to high glucose stimulation in pancreatic β-cells. The results that high glucose-induced depolarization is inhibited by dymanin inhibition indicate that the reduction of channel density by endocytosis is crucial for reduction of K_{ATP} currents that leads to membrane

depolarization (Fig. 2). The observation that 1 hr incubation in 17G in the presence of dynasore does not induce further depolarization (Fig. 2C) strongly suggests that channel blockade by the increase in intracellular ATP concentrations in high glucose conditions does not sufficiently reduce K_{ATP} currents when the surface channel density remains unchanged. However, my study does not entirely exclude the possibility that the increase in intracellular ATP concentrations in response to high glucose stimulation also contributes to the reduction of K_{ATP} currents. In order to investigate this issue, quantitative analysis of intracellular ATP concentration changes in response to high glucose stimulation will be required.

The second question I asked was the signaling mechanism of how high glucose facilitates K_{ATP} channel endocytosis. Considering that the surface density of a membrane protein is determined by the balance between the rate of forward trafficking and the rate of endocytosis, and that AMPK signaling promotes K_{ATP} channel trafficking to the plasma membrane (Lim et al., 2009; Park et al., 2013), inhibition of AMPK at high glucose stimulation may result in the increase in surface channel density. To test this possibility, I conducted RMP recordings with compound C, AMPK inhibitor, and I observed that AMPK inhibition alone does not induce membrane depolarization in a short time (Fig. 4B), suggesting that AMPK inhibition is not enough but high glucose may activate another signal pathway to facilitate endocytosis of K_{ATP} channels rapidly. As a possibility, I first examined mTOR signaling, but mTOR inhibitor rapamycin did

not affect β-cell response to high glucose (Fig. 4A). I then tested the involvement of PKC in high glucose-induced endocytosis of K_{ATP} channels.

As I mentioned earlier, roles of PKC in the regulation of K_{ATP} currents have been intensively investigated, but results are rather complicated. Acute application of PMA, a PKC activator, was shown to increase K_{ATP} currents by increasing the open probability of the channels by decreasing ATP sensitivity (Light *et al*., 2000), whereas prolonged incubation in PMA induces decrease in K_{ATP} current by promoting channel endocytosis (Hu K *et al*, 2003; Aziz *et al*., 2011). Inhibition of K_{ATP} currents by acute application of PMA was also reported, but it was observed in vascular smooth muscles (Bonev and Nelson, 1996). My results indicated that the PKC plays crucial roles in promoting endocytosis of K_{ATP} channels and depolarization in response to high glucose stimulation. In spite that the involvement of PKC in K_{ATP} channel endocytosis was reported in previous studies using ventricular myocytes or heterologous expression systems (Hu K *et al*, 2003; Aziz *et al*., 2011), PMA or carbachol does not induce endocytosis of endogenous K_{ATP} channels or depolarization in INS-1 cells at least within 1 hr (Fig. 7). I do not have a clear idea to explain the specific nature of PKC signaling activated by high glucose. Considering the diversity of PKC isoforms, high glucose may possibly activate a specific isoform of PKC in β-cells, which is distinguished from PKC isoforms activated by carbachol or PMA. Such possibility needs to be investigated in future studies.

Actually, while I asked which signaling pathway is involved in high glucose-induced endocytic trafficking of K_{ATP} channels in INS-1 cells, I firstly examined PLC inhibitor (U73122). But U73122 depolarized RMP by itself before applying high glucose solutions. Thus, I could not investigate effect of PLC inhibition on high glucose-induced depolarization anymore. Based on the previous report that U73122 inhibits acetylcholine-activate K^+ currents by inhibiting PIP2-channel interaction independently of PLC inhibiting effects (Cho *et al.*, 2001), and that K_{ATP} channels are also PIP2-dependent channels, it is likely that U73122 inhibits K_{ATP} channels by the same mechanism. However, this possibility needs to be investigated in future studies.

My study contradicts to the previous report showing that 17 mM glucose recruits the K_{ATP} channels to the plasma membrane and increase K_{ATP} currents in a Ca^{2+} and PKA-dependent manner (Yang *et al*, 2007). It is well known that β-cells, when subjected to initial glucose stimulation long enough to lead second-phase insulin secretion, release more insulin when re-exposed to glucose. Such glucose sensitization is referred to as 'β-cell memory' to glucose stimulation (Grill et al., 1978; Grodsky, 1972; Nesher and Cerasi, 2002; Straub and Sharp, 2002; Zawalich and Zawalich, 1996). So, Yang et al (2007) proposed that recruitment of K_{ATP} channels by high glucose may increase the β-cell responsiveness to next glucose stimulation and thereby contribute to 'β-cell memory' (Nesher *et al*, 1989). Since treatment time in 17G in their experiments was 1

hr, I wondered whether long-term effect was different from the acute effect of high glucose stimulation. So, I increased exposure time in 17G from 5 min to 1 h and performed immunocytochemistry after cell fixation. But I didn't see the clear recruit phenomenon on the surface even after 1 hr treatment with 17G (data no shown). At this moment, I do not have a clue to explain the discrepancy between two studies.

In addition, I investigated the involvement of cytoskeletons and microtubules in the endocytosis of K_{ATP} channels. I observed that pretreatment of INS-1 cells with taxol (microtubule stabilizer) and phalloidin (microfilament stabilizer) inhibited high glucose-induced depolarization (data not shown), but I could not confirm whether these compounds indeed inhibit K_{ATP} channel endocytosis. It requires more detailed examination.

I also tested whether released insulin particles cause endogenous K_{ATP} channel regulation and $P2Y_1$ receptor is involved in PKC-mediated 17 mM glucose response, as was previously suggested (Wuttke *et al*, 2013). To this end, I conducted the RMP recording with MRS 2179 ($P2Y_1$ receptor blocker) pretreatment in INS-1 cell, and I could not find special association with $P2Y_1$ receptor in 17 mM glucose response (data not shown).

Taken together, my study reveals that the glucose-stimulated depolarization of β-cell membrane potential, which is an initial step of glucose-stimulated insulin secretion, is driven by endocytosis of K_{ATP} channels. Contribution of channel inhibition by intracellular ATP to the glucose-stimulated depolarization needs to be re-evaluated.

Molecular mechanisms underlying glucose-stimulated endocytosis of K_{ATP} channels are not fully elucidated, but the involvement of PKC is suggested. To further explore the mechanism for PKC-induced channel endocytosis and its regulation, intensive experiments will be required to demonstrate directly the contribution of this mechanism to β-cell function in physiological and pathological contexts. Possibly, dysregulation of PKC pathway may alter endocytic trafficking of K_{ATP} channels to cause dysregulation of insulin secretion. I think my study lays the foundation for future work on K_{ATP} channel regulation in pancreatic β-cells.

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ABSTRACT in KOREAN

췌장베타 세포에 있는 ATP 민감성 포타슘 (K_{ATP} channel) 이온 통로는 인슐린 분비 조절을 비롯하여 포도당과 에너지 공급 등 대사 과정의 중추적인 역할을 맡고 있다. 나는 이번 연구에서 세포 굶김 상태 (glucose deprivation)가 AMP 활성단백질 인산화 효소 (AMPK)를 통해 췌장세포막을 과분극 (hyperpolarization) 상태로 만들며 ATP 민감성 포타슘 이온통로들이 세포막에 모이도록 한다는 사실을 재현하였고, 이 상태를 나의 실험 초기 조건으로 삼아 연구를 진행하였다. 그리고 고농도의 포도당 (17 mM) 이 유입되면 이전까지 알려진 바와는 달리 ATP 민감성 포타슘 이온통로의 채널 막힘 현상이 아닌, 세포 내부로 이동하는 현상이 포도당 유도성 인슐린 분비현상 (GSIS – glucose stimulate insulin secretion) 에 더욱 중요하게 작용한다는 새로운 사실을 발견하였다. 이 이온통로의 억제현상은 세포막의 탈분극 (depolarization) 을 유도, 일련의 과정들을 거쳐 인슐린 분비가 일어나게 되는데 이는 포유류 생체내의 에너지 대사에 매우 중요한 역할을 한다.

나는 고농도의 포도당을 신호로, ATP 민감성 포타슘 이온통로가 세포막 함입과정 (endocytosis) 을 거쳐 세포 내부로 들어오는 데에 다이나민 (dynamin) 단백질이 관여하고 있다는 사실을 알게 되었고 무엇보다도 이 세포막 함입 과정에 PKC (Protein Kinase C) 가 핵심 역할을 담당하고 있음을 증명하였다. 그리고 포도당 유래 ATP 유도성 채널억제현상과 채널의 세포 내 합임현상 두 가지 가능성을 함께 염두 해두고 관련 실험들을 계속 진행하였다.

중심 단어: ATP 민감성 포타슘 이온통로 (K_{ATP} channel), 췌장베타세포, AMP 활성 단백질 인산화 효소 (AMPK), 포도당 (glucose), 탈분극 (depolarization), 과분극 (hyperpolarization), 세포막 함입과정 (endocytosis), 세포 굶김 (glucose deprivation), 다이나민 (dynamin), PKC (protein kinase C), 세포골격 (cytoskeleton)

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