Published in final edited form as:

Endocrine. 2014 November ; 47(2): 463–471. doi:10.1007/s12020-013-0149-y.

Contribution of Protein Kinase Ca in the Stimulation of Insulin by the Down Regulation of $\text{Ca}_v\beta$ subunits

Senthilkumar Rajagopal¹, Blanche L. Fields, and Ganesan L. Kamatchi²

Department of Biology, Norfolk State University, Norfolk, VA 23504

Abstract

Voltage-gated calcium (Ca_v) channels and protein kinase C (PKC) isozymes are involved in insulin secretion. In addition, $\text{Ca}_v\beta$, one of the auxiliary subunits of Ca_v channels, also regulate the secretion of insulin as knockout of $\text{Ca}_v\beta 3$ ($\beta 3^{-/-}$) subunits in mice led to efficient glucose homeostasis and increased insulin levels. We examined if other types of $\text{Ca}_v\beta$ subunits also have similar properties. In this regard, we used small interfering RNA (siRNA) of these subunits (20 μg each) to down regulate them and examined blood glucose, serum insulin and PKC translocation in isolated pancreatic β cells of mice. While the down regulation of $\text{Ca}_v\beta 2$ and $\beta 3$ subunits increased serum insulin levels and caused efficient glucose homeostasis, the down regulation of $\text{Ca}_v\beta 1$ and $\beta 4$ subunits failed to affect both these parameters. Examination of PKC isozymes in the pancreatic β -cells of $\text{Ca}_v\beta 2$ or $\beta 3$ siRNA injected mice showed that three PKC isozymes, viz., PKC α , β II and θ translocated to the membrane. This suggests that when present, $\text{Ca}_v\beta 2$ and $\beta 3$ subunits inhibited PKC activation. Among these three isozymes, only PKC α siRNA inhibited insulin and increased glucose concentrations. It is possible that the activation of PKCs β II and θ are not sufficient for the release of insulin and PKC α is the mediator of insulin secretion under the control of $\text{Ca}_v\beta$ subunits. Since $\text{Ca}_v\beta$ subunits are present intracellularly, it is possible that they i) inhibited the translocation of PKC isozymes to the membrane and ii) decreased the interaction between Ca_v channels and PKC isozymes and thus the secretion of insulin.

Keywords

$\text{Ca}_v\beta$ subunits; PKC α ; Insulin; Calcium Channels

²Corresponding Author: Ganesan L. Kamatchi, Department of Biology, Norfolk State University, 700 Park Avenue, Norfolk, VA 23504, Ph: 757-823-2327, Fax: 757-823-2000.

¹Present Address: Department of Zoology, Nizam College, Osmania University, Hyderabad – 500 001, Andhra Pradesh, India., Ph: 01-040-23234231, Fax: 01-040-232400806

Declaration of Interest

The authors fully declare any financial or other potential conflict of interest.

The content is solely the responsibility of the authors and doesn't necessarily represent the views of the National Institute of Child Health and Human Development or the National Institutes of Health.

Authorship Contributions

Participated in research design: Kamatchi, GL and Rajagopal S.

Conducted experiments: Rajagopal S and Fields, BL.

Performed data analysis: Kamatchi, GL and Rajagopal S.

Wrote or contributed to the writing of the manuscript: Kamatchi, GL and Rajagopal S.

Other: Kamatchi acquired funding for the research.

Introduction

Glucose is the major stimulant for the secretion of insulin from the β -cells of the pancreas. Following its entry into the cells, it is metabolized to ATP leading to the increase of ATP-to-ADP ratio. As a consequence, ATP-sensitive K^+ channels close, cell membranes depolarize and the high voltage-gated calcium (Ca_v) channels open. The subsequent entry of Ca^{2+} into the cells increases intracellular Ca^{2+} [Ca^{2+}]_i, activates protein kinase C (PKC) isozymes and releases insulin [1–4]. In addition, glucose also activates PKC directly leading to the insulin release [5,6].

The family of PKC consists of eleven isozymes and grouped under three subfamilies, viz., classic PKCs (α , βI , βII and γ), novel PKCs (δ , ϵ , η and θ) and atypical PKCs (ζ , ι/λ , and μ) based on cofactor requirements [7]. PKCs α and ϵ are dominant in the pancreatic islet β -cells and translocate to membrane by the stimulation with glucose [5, 8–10]. The contribution of other PKC isozymes cannot be ruled out as PKCs βII , γ , θ , λ and ζ also were translocated to membrane with glucose or potassium-induced stimulation of insulin secretion [6, 10, 11].

Ca_v channels are a family of hetero-multimers classified into ten types (Ca_v 1.1, 1.2, 1.3, 1.4; 2.1, 2.2, 2.3 and 3.1, 3.2, 3.3) based on their major and the pore-forming α_1 subunits. In addition to this, there are two to three minor or auxiliary subunits, viz., β , α_2/δ and γ that modulate the channel expression and current kinetics [12, 13]. While Ca_v 1.2 and 1.3 type channels play a major role in the release of insulin, other types of Ca_v channels also contribute to this process to a certain extent [14–19]. These channels interact with the PKC isozymes and that subsequently leads to the exocytosis of insulin [20–22]. The interaction between these two proteins is made possible as PKC isozymes target the serine/threonine (Ser/Thr) sites of $Ca_v\alpha_1$ subunits leading to the potentiation of the Ca_v currents (I_{Ca}) [23–28].

In spite of the interaction between the $Ca_v\alpha_1$ subunits and the PKC isozymes in the insulin release, $Ca_v\beta$ subunits also modulate insulin secretion. Recently, in a classic study, increased insulin release and glucose homeostasis were observed in $Ca_v\beta 3$ knockout ($\beta 3^{-/-}$) mice [29]. It is not known if these activities are applicable to $Ca_v\beta 2$, another $Ca_v\beta$ subunit present in the pancreas [4, 29–32] among the four ($\beta 1$ to $\beta 4$) $Ca_v\beta$ subunits known. It is one of our aims to examine the effects of $Ca_v\beta 2$ subunit on insulin release and glucose homeostasis.

The increased insulin secretion and glucose homeostasis in $\beta 3^{-/-}$ mice were accompanied with enhanced inositol triphosphate (IP3), mobilization of [Ca^{2+}]_i and Ca^{2+} oscillation frequency [29]. Hence, when present, these components, along with insulin release, may have been inhibited by the $Ca_v\beta 3$ subunit, a process called negative modulation. The negative modulation of IP3 and [Ca^{2+}]_i may have inhibited the activation of PKC [7, 33] and subsequently insulin release as PKC is a known secretagogue for insulin [2, 20]. However, it is not clear which PKC isozymes are involved in this process. Hence our second aim in this study is to identify the PKC isozymes involved in the negative modulation of insulin release by the $Ca_v\beta$ subunits. In this regard, we employed small interfering RNA (siRNA) to down regulate $Ca_v\beta$ subunits and studied the blood glucose, serum insulin and PKC translocation in isolated pancreatic β cells of mice.

Materials

Animals

Adult male ICR mice (25–30 gm; Harlan Laboratories, Inc., USA) were used for all the studies. They were housed at a constant temperature of 22–23 °C with 12h light/dark cycle and *ad libitum* access to food and water unless otherwise indicated. All animal experiments were conducted at Old Dominion University, Norfolk, Virginia, following the stipulations set by their Institute Animal Care and Use Committee (IACUC).

siRNAs and their injection

The siRNAs (unmodified) for the $\text{Ca}_v\beta$ subunits were obtained commercially (Sigma, St. Louis, MO, USA) and their sequence given by the supplier is as follows: $\text{Ca}_v\beta 1$, 5' GCC UUA GCC CAG CUC GAG 3' and 5' UCU CGA GCU GGG CUA AAG 3'; double stranded RNA oligoribonucleotide NNGCGCGCUUUGUAGGAUUCA (5'-3') was used as a control siRNA. $\text{Ca}_v\beta 2$, 5' CAA CGA AGC CGG CAU AAA 3' and 5' AUU UAU GCC GGC UUC GUU 3'; scrambled $\text{Ca}_v\beta 2$, 5' AGC CGG CAC AAG AUA AAU 3' and 5' AUU GUA UCU UGU GUG CCG GCU 3'; $\text{Ca}_v\beta 3$, 5' GUG AGA UUG AGC GCA UAU 3' and 5' AAU AUG CGC UCA AUC UCA 3'; scrambled $\text{Ca}_v\beta 3$, 5' CUG GUA CUU AGG GAA UUG 3' and 5' CAA UUC CCU AAG UAC CAG 3' and $\text{Ca}_v\beta 4$, 5' GGU UAG AGC UGA AAC CUC A 3' and 5' UGA GGU UUC AGC UCU AAC C 3'; double stranded RNA oligoribonucleotide NNGCGCGCUUUGUAGGAUUCA (5'-3') was used as a negative control.

The siRNAs for the selected PKC isozymes (α , βII and θ) were prepared in our laboratory (Life Technologies, Grand Island, NY, USA). The sense and antisense DNA templates respectively for the preparation of siRNAs are as follows: PKC α , 5' CAG CCC AAC ATT TCC TGT CTC 3' and 5' TGG GCT GCC ATA GCC TGT CTC 3'; scrambled PKC α , 5' ATC AGC ACC ATA CCC TGT CTC 3' and 5' ACG ACT AGG CTT TCC TGT CTC 3'; PKC βII , 5' GAA CTT CGA CAA GCC TGT CTC 3' and 5' GAA GTT CTC AGC ACC TGT CTC 3'; scrambled PKC βII , 5' ACT ACA TCA GTA GCC TGT CTC 3' and 5' ATG TAG TCT CAC GCC TGT CTC 3'; PKC θ , 5' GCT GAA ACC TCA AGG CCG AAT 3' and 5' ATT CGG CCA TGA GGT TTC AGC 3'; scrambled PKC θ , 5' GAT AGA TCC CAA GCC GGA ATC 3' and 5' TAG GTC CTG GAT CGA CTT GAC 3'. The siRNA aliquots were stored at –20 °C in a final concentration of 100 μM . They were used at a concentration of 20 $\mu\text{g}/\text{mice}$ by suspending in 1 ml of normal saline; this was injected rapidly ('high-pressure' injection; <5 seconds) into the tail vein of the mice. The mice were used 24 hours post injection for 1) GTT, 2) isolation of pancreatic islets and insulin determination, 3) islet cell culture, siRNA transfection, insulin determination and immunocytochemistry and 4) Western blotting.

GTT

The mice were deprived of food for 12–14h before the GTT, but had free access to water. The GTT was initiated with the injection of D-glucose (2 mg/g body weight, i.p.). The blood for GTT and insulin determination was taken at 0 min (before glucose injection) and 15, 30 and 60 min (after the administration of glucose) from the tail veins of these mice. The

glucose concentration was determined using the Glucometer Elite (Bayer Corp., Diagnostics GmbH, Leverkusen, Germany) and insulin by ELISA (Mouse Ultra-sensitive Insulin Immunoassay Kit, Alpco Diagnostics, Inc. Salem, NH, USA) following the protocol given by the supplier.

Isolation of Pancreatic Islets and Insulin Determination

The mouse was euthanized according to a protocol approved by the IACUC of Old Dominion University. The pancreatic islets were isolated following the method published [49]. They were incubated at 37°C overnight to allow them to recover from the collagenase treatment before the beginning of any experiment.

Three size-matched islets per group were manually selected, incubated in Krebs-Ringer solution, and stimulated at 37 °C with glucose (16.7 mM) in a volume of 1 ml for 1 hour. The islets were then collected by centrifugation and the supernatant was assayed for insulin content as described above.

Islet Cell Culture, siRNA Transfection, Insulin Determination and Immunocytochemistry

Islet cell cultures obtained from naïve mice as described before [49], were used to transfect with siRNA and study insulin secretion. The islet cell suspension containing approximately 20,000 cells were seeded in Petri dishes containing cover slips coated with polylysine (0.5 mg/ml; Sigma). The cultures were used for the transfection with the siRNA and cDNA following the protocol given by the supplier (Reverse transfection; Lipofectamine RNAiMAX, Invitrogen, Carlsbad, CA, USA) after 24 hrs. The combinations for the transfection were, 1) scrambled or $Ca_v\beta$ subunit siRNA (5 µg/dish), 2) $Ca_v\beta$ subunit siRNA (2.5 µg) and the PKC isozyme cDNA (2.5 µg) subcloned into green fluorescent protein (GFP) containing vector or 3) selected PKC isozyme siRNA (2.5 µg) and PKC isozyme cDNA (2.5 µg) subcloned into GFP containing vector.

Insulin release was studied approximately 48 hrs after the transfection by exposing the cells to 16.7 mM glucose for 1hr. The supernatant from the dishes was assayed for insulin content by ELISA as described above.

Translocation of PKC isozymes was studied in cultured pancreatic islet cells of mice by immunocytochemistry using a Carl Zeiss AG 510 LSM confocal microscope and a $\times 100$ Plan-Apochromat $\times 100/1.4$ oil objective. After the fixation of the cells with 4% paraformaldehyde and permeabilization with 0.1% Triton-X-100, the cells were incubated with rabbit polyclonal antibodies for specific PKC isozymes (Santa Cruz, CA, USA), diluted in 10% serum/1X PBS (1:100) for overnight at 4 °C. Cover slips were washed three times in 1% serum for 5 min each, incubated for 1 h with 1:2000 dilution of Alexa Fluor 488 goat anti rabbit IgG (Invitrogen) diluted in 10% serum/1X PBS. The excitation was measured by multi-track mode using the 543-nM and 633-nM lines of the HeNe lasers, and the emitted light was collected using greater than 560-nM and greater than 650-nM long-pass filters, respectively.

Western Blotting of the Pancreatic Islets

The silencing of the $\text{Ca}_v\beta$ subunits by the siRNA was studied by Western blotting. The islets were pooled (~500 in each group) and equilibrated with 150 μl of homogenization buffer [in mM: EGTA 2, EDTA 2 and Tris-HCl 20 (pH 7.4), 2-mercaptoethanol 10 and protease inhibitors (1 mM phenyl-methylsulfonyl fluoride, 0.3 μM aprotinin and leupeptin (20 $\mu\text{g}/\text{ml}$)] and sonicated. The cytosolic (supernatant) and membrane (pellet) fractions were prepared by centrifugation at $100,000 \times g$ for 1 h at 4 °C. The pellet was resuspended (150 μl) in the homogenization buffer containing 0.1% triton $\times -100$. Protein fractions (100 $\mu\text{g}/\text{lane}$) were analyzed on SDS/polyacrylamide gel with a Laemmli buffer system and transferred to nitrocellulose membrane. Immunodetection of $\text{Ca}_v\beta$ subunit proteins was performed with specific polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA and EMD Biosciences Inc., Darmstadt, Germany). The proteins were identified with ECL detection kit (Amersham Life Sciences, Buckinghamshire, UK) as instructed by the manufacturer.

Results

We employed siRNA for the down regulation of the $\text{Ca}_v\beta$ subunits. The siRNA was injected through the tail vein by high pressure (meaning the shortest possible time), a method known to increase the potency of the genetic material. The rapid injection of unmodified genetic material into the tail vein, in a large volume, has been successfully used by several laboratories to deliver plasmid DNA [34, 35] or RNA [36–38]. According to Song et al. [37], the target protein was reduced nearly to the background and the effect persisted for 10 days (with 3 consecutive daily injections). The study by Lewis et al. [36] showed that the expression of firefly luciferase was down regulated by 80–90% 24 hours after the injection of siRNA-*luc*⁺. Hamar et al. [38] identified a reduction of *Fas* mRNA by $74 \pm 8\%$ in 24 hours after the injection of *Fas* siRNA. Similarly, in this study also both $\text{Ca}_v\beta 2$ and $\beta 3$ subunits were down regulated by the respective siRNAs as shown in the Western blotting (Fig. 1). However, both $\text{Ca}_v\beta 1$ and $\beta 4$ subunits were absent even in the control, suggesting that these two $\text{Ca}_v\beta$ subunits are not present in the pancreatic islets.

The absence of $\text{Ca}_v\beta 1$ and $\beta 4$ subunits reflected in the glucose tolerance test (GTT) as both blood glucose and serum insulin levels were not altered with the injection of the siRNA of either of these two subunits (Fig. 1). In contrast, blood glucose levels were decreased and serum insulin concentration increased in the groups injected with the siRNA for $\text{Ca}_v\beta 2$ or $\text{Ca}_v\beta 3$ subunits. It is significant that in both these groups, fasting blood glucose or insulin levels were not affected. The changes seen in the insulin levels of intact animals with $\text{Ca}_v\beta$ subunit siRNA were reproduced by the *in vitro* studies also. Stimulation of the isolated islets from the $\text{Ca}_v\beta 1$ siRNA injected mice with 16.7 mM glucose did not increase insulin levels (Fig. 2A). In contrast, insulin levels were increased by the above concentration of glucose from the islets of $\text{Ca}_v\beta 2$ or $\beta 3$ subunit siRNA injected mice. In these studies also, the basal insulin levels were not increased. These results were reproduced in the cultured pancreatic β -cells transfected with the siRNA. While $\text{Ca}_v\beta 1$ siRNA transfected cells failed to show any increase in the insulin, $\text{Ca}_v\beta 2$ or $\beta 3$ siRNA transfected cells showed increase in the insulin level with the stimulatory concentration of glucose (Fig. 2B).

Based on the role of $\text{Ca}_v\beta 2$ and $\beta 3$ subunits in the increased glucose homeostasis and insulin levels, we studied the translocation of PKC isozymes to examine if they are involved in this process in the mice administered $\text{Ca}_v\beta 2$ or $\beta 3$ siRNA. Confocal microscopy of the cultured β cells was conducted employing pGFP tagged PKC isozymes. Of the eleven isozymes (PKCs α , βI , βII , γ , δ , ϵ , η , θ , ζ , ι/λ and μ) that we examined, only PKCs α , βII and θ were translocated to the membrane by either $\text{Ca}_v\beta 2$ or $\beta 3$ siRNA as indicated by the intensity of the membrane in figure 3.

The role of PKCs α , βII and θ was followed up by studying their involvement in the insulin release and glucose homeostasis. In this regard, instead of activating these isozymes, (and studying the possibly increased insulin and decreased glucose levels), we opted for their down regulation with siRNA and studying the likelihood of decreased insulin and increased glucose levels by GTT. Surprisingly of the three siRNAs that we examined, only PKC α siRNA decreased insulin concentration and increased blood glucose level and both PKC βII and θ siRNAs failed to affect these two parameters (Fig. 4). Subsequently, the efficacy of the PKC α , βII and θ siRNAs to down regulate these PKC isozymes was determined by confocal microscopy (Fig. 5). The siRNAs that we employed specifically down regulated the isozymes intended and not the isozymes of the other selected PKC subtypes. To be specific, PKC α siRNA down regulated PKC α isozyme, but not PKC γ or ϵ ; PKC βII siRNA down regulated PKC βII isozyme and not PKCs γ and θ ; PKC θ siRNA down regulated PKC θ and didn't cross react with PKCs βII and ϵ (Fig. 5).

Discussion

A cascade of intermediaries is involved in the secretion of insulin from the pancreatic β -cells. $[\text{Ca}^{2+}]_i$ and PKC are parts of this cascade and contribute at various stages of this pathway. The concentration of $[\text{Ca}^{2+}]_i$ is determined by the entry of Ca^{2+} into the cells through the Ca_v channels. Pancreatic β -cells harbor seven members of Ca_v channel family including 1.2, 1.3, 2.1, 2.2, 2.3, 3.1 and 3.2 [4, 39]. Among these, $\text{Ca}_v 1.0$ subfamily of channels is responsible for 60–80% of glucose-stimulated insulin secretion [4, 39]. The remaining 20–40 % of insulin secretion is under the control of $\text{Ca}_v 2.0$ family of channels, though the contribution by the $\text{Ca}_v 2.2$ type of channels is doubtful [14–19, 40].

In addition to the Ca_v channels as such, $\text{Ca}_v\beta$ subunits also modulated the secretion of insulin as there was increased insulin and efficient glucose homeostasis in $\beta 3^{-/-}$ mice [29]. The effects seen in $\beta 3^{-/-}$ mice were reproduced by the down regulation of the 3 subunits with the siRNA in this study (Fig. 1). The negative modulation of insulin secretion by the $\text{Ca}_v\beta 3$ subunits was shared by the $\text{Ca}_v\beta 2$ subunits, the only other $\text{Ca}_v\beta$ subunit present in the pancreas [4, 29–32]. The modulation of insulin secretion by the $\text{Ca}_v\beta 2$ and $\beta 3$ siRNA may be coming from the pancreas itself as the down regulation of $\text{Ca}_v\beta 1$ or $\beta 4$ subunits, the β subunits that are not present in the pancreas [4, 29–32] failed to reproduce this effect. Direct effect at the level of pancreas is further supported as glucose stimulation of i) isolated islets from $\text{Ca}_v\beta 2$ or $\beta 3$ siRNA injected mice and ii) cultured pancreatic β -cells transfected with $\text{Ca}_v\beta 2$ or $\beta 3$ siRNA led to increased insulin release (Fig. 2).

$\text{Ca}_v\beta 2$ - or $\beta 3$ siRNA-induced insulin release may be due to the activation of PKC isozymes (Fig. 3). This may have come indirectly by the increased $[\text{Ca}^{2+}]_i$ and IP3 levels with the down regulation of $\text{Ca}_v\beta 2$ or $\beta 3$ subunits as observed in the pancreatic β -cells of $\beta 3^{-/-}$ mice [29]. It is well known that these two intermediaries are the known activators of certain PKC isozymes [7, 33]. PKCs are known secretagogues for insulin even when $[\text{Ca}^{2+}]_i$ in β -cells is very low and they could substitute for Ca^{2+} as triggering signal for insulin release [2, 20]. In addition, PKC isozymes also mediate Ca^{2+} -stimulated insulin exocytosis [2]. Taken together, when present, $\text{Ca}_v\beta 2$ and $\beta 3$ subunits negatively modulated $[\text{Ca}^{2+}]_i$, IP3 and PKC, leading to the inhibition of PKC isozymes and insulin secretion. The inhibition of PKC activity by the $\text{Ca}_v\beta 3$ subunits is known as increased neuronal function, a measure of PKC activation, was observed in $\text{Ca}_v\beta 3$ null mice [41].

PKCs βII and θ (in addition to PKC α) translocated to the membrane of pancreatic β -cells in this study as seen by others before [10]. However, the failure of PKC βII and θ to modulate insulin secretion suggests that either one of these two isozymes *per se* is not sufficient for insulin secretion involving $\text{Ca}_v\beta$ subunits. On the contrary, PKC α , the predominant cPKC isozymes in the pancreatic β -cells [5, 8–10] decreased insulin and increased glucose levels upon its down regulation in this study (Fig. 4). This suggests that when present, PKC α isozyme may be involved in insulin secretion. Support for the role of PKC α in insulin release may be derived from the muscarinic cholinergic system, a well-known regulator of glucose-dependent insulin secretion [42, 43]. The muscarinic cholinergic system may release insulin through PKC α as acetyl- β -methylcholine (MCh), an agonist of muscarinic cholinergic receptors activated PKC α [44]. PKC α isozyme may have targeted $\text{Ca}_v 1.0$ family of channels as they are a good substrate for numerous protein kinases, such as protein kinase A, PKC, cGMP-dependent protein kinase, calcium/calmodulin-dependent kinase II and protein tyrosine kinases [3, 39].

$\text{Ca}_v\beta$ subunits are members of membrane associated guanylate kinase family, thereby suggesting a role in scaffolding multiple signaling pathways around the channel. The tridimensional structure of this subunit supports the above statement as it has large space for the interaction with putative partners [39]; PKC α may be one such partner. It was observed in our laboratory that $\text{Ca}_v\beta$ subunits inhibited the modulation of I_{Ca} by MCh and phorbol 12-myristate 13-acetate (unpublished observation). It has been reported by others also that PKC responsiveness of the I_{Ca} was modulated by $\text{Ca}_v\beta$ subunits in a $\text{Ca}_v\alpha 1$ subunit-dependent manner [24, 45–48]. Taken together, insulin secretion is the result of interaction between $\text{Ca}_v\alpha 1$ subunits, their Ser/Thr sites, $\text{Ca}_v\beta$ and PKC isozymes. The identification of the roles of these components will not only be significant for the understanding of the intricacies of the insulin secretion but also for the ion channel physiology.

Acknowledgments

Funding

This work was supported by National Institutes of General Medical Sciences (SCORE3, GM096947) to G.L.K. The project described was supported in part by Grant Number G11HD052382 from the National Institute of Child Health and Human Development.

References

1. Ashcroft FM, Rorsman P. Electrophysiology of the pancreatic β -cells. *Prog Biophys Mol Biol*. 1989; 54:87–143. [PubMed: 2484976]
2. Straub S, Sharp G. Glucose-stimulated signaling pathways in biphasic insulin secretion. *Diabetes Metab Res*. 2002; 18:451–463.
3. Yang SN, Berggren PO. Cav2.3 channels and PKC lambda: new Players in insulin secretion. *J Clin Invest*. 2005; 115:16–20. [PubMed: 15630435]
4. Yang S, Berggren PO. β -cell Cav channel regulation in physiology and pathophysiology. *Am J Physiol-Endoc M*. 2005; 288:E16–E28.
5. Yedovitzky M, Mochly-Rosen D, Johnson J, Gray M, Ron D, Abramovitch E, Cerasi E, Nesher R. Translocation inhibitors define specificity of protein kinase C isozymes in pancreatic β -cells. *J Biol Chem*. 1997; 272:1417–1420. [PubMed: 8999804]
6. Hashimoto N, Kido Y, Uchida T, Matsuda T, Suzuki K, Inoue H, Matsumoto M, Ogawa W, Maeda S, Fujihara H, Ueta Y, Uchiyama Y, Akimoto K, Ohno S, Noda T, Kasuga M. PKC λ regulates glucose-induced insulin secretion through modulation of gene expression in pancreatic β cells. *J Clin Invest*. 2005; 115:138–145. [PubMed: 15630453]
7. Newton A. Diacylglycerol's affair with protein kinase C turns 25. *Trends Pharmacol Sci*. 2004; 25:175–177. [PubMed: 15116719]
8. Knutson K, Hoenig M. Identification and subcellular characterization of protein kinase-C isoforms in insulinoma beta-cells and whole islets. *Endocrinology*. 1994; 135:881–886. [PubMed: 8070382]
9. Wan QF, Dong Y, Yang H, Lou X, Ding J, Xu T. Protein kinase activation increases insulin secretion by sensitizing the secretory machinery to Ca^{2+} . *J Gen Physiol*. 2004; 124:653–662. [PubMed: 15572345]
10. Zhang H, Nagasawa M, Yamada S, Mogami H, Suzuki Y, Kojima I. Bimodal role of conventional protein kinase C in insulin secretion from rat pancreatic β -cells. *J Physiol (Lond)*. 2004; 561:133–147. [PubMed: 15388777]
11. Pinton P, Tsuboi T, Ainscow E, Pozzan T, Rizzuto R, Rutter G. Dynamics of glucose-induced membrane recruitment of protein kinase C β III in living pancreatic islet β -cells. *J Biol Chem*. 2002; 277:37702–37710. [PubMed: 12149258]
12. Dolphin AC. Calcium channel diversity: multiple roles of calcium channel subunits. *Curr Opin Neurobiol*. 2009; 19(3):237–244. [PubMed: 19559597]
13. Catterall WA. Signaling complexes of voltage-gated sodium and calcium channels. *Neurosci Lett*. 2010; 486(2):107–116. [PubMed: 20816922]
14. Davalli A, Biancardi E, Pollo A, Soggi C, Pontiroli A, Pozza G, Clementi F, Sher E, Carbone E. Dihydropyridine-sensitive and -insensitive voltage-operated calcium channels participate in the control of glucose-induced insulin release from human pancreatic β cells. *J Endocrinol*. 1996; 150:195–203. [PubMed: 8869586]
15. Ligon B, Boyd A, Dunlap K. Class A calcium channel variants in pancreatic islets and their role in insulin secretion. *J Biol Chem*. 1998; 273:13905–13911. [PubMed: 9593738]
16. Sher E, Giovannini F, Codignola A, Passafaro M, Giorgi-Rossi P, Volsen S, Craig P, Davalli A, Carrera P. Voltage-operated calcium channel heterogeneity in pancreatic β cells: physiopathological implications. *J Bioenerg Biomembr*. 2003; 35:687–696. [PubMed: 15000528]
17. Schulla V, Renstrom E, Feil R, Feil S, Franklin I, Gjinovci A, Jing X, Laux D, Lundquist I, Magnuson M, Obermuller S, Olofsson C, Salehi A, Wendt A, Klugbauer N, Wollheim C, Rorsman P, Hofmann F. Impaired insulin secretion and glucose tolerance in beta cell-selective Cav1.2 Ca^{2+} channel null mice. *EMBO J*. 2003; 22:3844–3854. [PubMed: 12881419]
18. Jing X, Li DQ, Olofsson C, Salehi A, Surve V, Caballero J, Ivarsson R, Lundquist I, Pereverzev A, Schneider T, Rorsman P, Renström E. Cav2.3 calcium channels control second-phase insulin release. *J Clin Invest*. 2005; 115:146–154. [PubMed: 15630454]
19. Muller YL, Hanson R, Zimmermann C, Harper I, Sutherland J, Kobes S, Knowler W, Bogardus C, Baier L. International Type 2 Diabetes 1q Consortium. Variants in the $\text{Ca}_v2.3$ ($\alpha 1E$) subunit of voltage-activated Ca^{2+} channels are associated with insulin resistance and type 2 diabetes in Pima Indians. *Diabetes*. 2007; 56:3089–3094. [PubMed: 17720895]

20. Komatsu M, Schermerhorn T, Aizawa T, Sharp G. Glucose stimulation of insulin release in the absence of extracellular Ca^{2+} and in the absence of any rise in intracellular Ca^{2+} in rat pancreatic islets. *P Natl Acad Sci USA*. 1995; 92:10728–10732.
21. Komatsu M, Schermerhorn T, Straub S, Sharp G. Pituitary adenylate cyclase-activating peptide, carbachol, and glucose stimulate insulin release in the absence of an increase in intracellular Ca^{2+} *Mol Pharmacol*. 1996; 50:1047–1054. [PubMed: 8863853]
22. Lee IS, Hur EM, Suh BC, Kim MH, Koh DS, Rhee IJ, Ha H, Kim KT. Protein kinase A- and C-induced insulin release from Ca^{2+} -insensitive pools. *Cell Signal*. 2003; 15:529–537. [PubMed: 12639716]
23. McHugh D, Sharp E, Scheuer T, Catterall WA. Inhibition of cardiac L-type calcium channels by protein kinase C phosphorylation of two sites in the N-terminal domain. *P Natl Acad Sci USA*. 2000; 97:12334–12338.
24. Kamatchi GL, Franke R, Lynch C III, Sando JJ. Identification of Sites Responsible for Potentiation of Type 2.3 Calcium Currents by Acetyl- β -methylcholine. *J Biol Chem*. 2004; 279(6):4102–4109. [PubMed: 14625305]
25. Fang H, Franke R, Patanavanich S, Lalvani A, Powell N, Sando JJ, Kamatchi GL. Role of α 12.3 subunit I–II linker sites in the enhancement of Cav 2.3 currents by phorbol 13-myristate 13-acetate. *J Biol Chem*. 2005; 280:23559–23565. [PubMed: 15840578]
26. Fang H, Patanavanich S, Rajagopal S, Yi X, Gill M, Sando JJ, Kamatchi GL. Inhibitory role of Ser-425 of the α 12.2 subunit in the enhancement of Cav2.2 currents by phorbol-12-myristate, 13-acetate. *J Biol Chem*. 2006; 281:20011–20017. [PubMed: 16704976]
27. Rajagopal S, Fang H, Oronce C, Jhaveri S, Taneja S, Dehlin E, Snyder S, Sando JJ, Kamatchi GL. Site-specific regulation of Cav 2.2 channels by protein kinase C isozymes β II and ϵ . *Neuroscience*. 2009; 159:618–628. [PubMed: 19167461]
28. Rajagopal S, Fang H, Lynch C III, Sando JJ, Kamatchi GL. Effects of isoflurane on the expressed Cav2.2 currents in *Xenopus* oocytes depend on the activation of protein kinase C δ and its phosphorylation sites in the Cav2.2 α 1 subunits. *Neuroscience*. 2011; 182:232–240. [PubMed: 21402126]
29. Berggren PO, Yang SN, Murakami M, Efanov A, Uhles S, Kohler M, Moede T, Fernstrom A, Appelskog I, Aspinwall C, Zaitsev S, Larsson O, de Vargas L, Fecher-Trost C, Weibgerber P, Ludwig A, Leibiger B, Juntti-Berggren L, Barker C, Gromada J, Freichel M, Leibiger I, Flockerzi V. Removal of Ca^{2+} channel β 3 subunit enhances Ca^{2+} oscillation frequency and insulin exocytosis. *Cell*. 2004; 119:273–284. [PubMed: 15479643]
30. Gao B, Sekido Y, Maximov A, Saad M, Forgacs E, Latif F, Wei M, Lerman M, Lee J, Perez-Reyes E, Bezprozvanny I, Minna J. Functional properties of a new voltage-dependent calcium channel α 2 δ auxiliary subunit gene (CACNA2D2). *J Biol Chem*. 2000; 275:12237–12242. [PubMed: 10766861]
31. Iwashima Y, Abiko A, Ushikubi F, Hata A, Kaku K, Sano H, Eto M. Downregulation of the voltage-dependent calcium channel (VDCC) β -subunit mRNAs in pancreatic islets of type 2 diabetic rats. *Biochem Biophys Res Co*. 2001; 280:923–932.
32. Mears D. Regulation of insulin secretion in islets of langerhans by Ca^{2+} channels. *J Membr Biol*. 2004; 200:57–66. [PubMed: 15520904]
33. Newton A. Protein kinase C: poised to signal. *Am J Physiol-Endoc M*. 2010; 298:E395–E402.
34. Zhang G, Budker V, Wolff J. High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. *Hum Gene Ther*. 1999; 10:1735–1737. [PubMed: 10428218]
35. Liu F, Song Y, Liu D. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther*. 1999; 6:1258–1266. [PubMed: 10455434]
36. Lewis D, Hagstrom J, Loomis A, Wolff J, Herweijer H. Efficient delivery of siRNA for inhibition of gene expression in postnatal mice. *Nat Genet*. 2002; 32:107–108. [PubMed: 12145662]
37. Song E, Lee SK, Wang J, Ince N, Ouyang N, Min J, Chen J, Shankar P, Lieberman J. RNA interference targeting Fas protects mice from fulminant hepatitis. *Nat Med*. 2003; 9:347–351. [PubMed: 12579197]

38. Hamar P, Song E, Kokeny G, Chen A, Ouyang N, Lieberman J. Small interfering RNA targeting Fas protects mice against renal ischemia-reperfusion injury. *P Natl Acad Sci USA*. 2004; 101:14883–14888.
39. Yang SN, Berggren PO. The role of voltage-gated calcium channels in pancreatic beta-cell physiology and pathophysiology. *Endocr Rev*. 2006; 27:621–676. [PubMed: 16868246]
40. Pereverzev A, Vajna R, Pfitzer G, Hescheler J, Klockner U, Schneider T. Reduction of insulin secretion in the insulinoma cell line INS-1 by overexpression of a $\text{Ca}_v2.3$ ($\alpha 1E$) calcium channel antisense cassette. *Eur J Endocrinol*. 2002; 146:881–889. [PubMed: 12039710]
41. Shiraiwa T, Kashiwayanagi M, Iijima T, Murakami M. Involvement of calcium channel $\beta 3$ subunit in olfactory signal transduction. *Biochem Biophys Res Co*. 2007; 355:1019–1024.
42. Gilon P, Henquin J. Mechanisms and physiological significance of the cholinergic control of pancreatic β -cell function. *Endocr Rev*. 2001; 22:565–604. [PubMed: 11588141]
43. Gautam D, Han S, Hamdan F, Jeon J, Li B, Li J, Cui Y, Mears D, Lu H, Deng C, Heard T, Wess J. A critical role for beta cell M_3 muscarinic acetylcholine receptors in regulating insulin release and blood glucose homeostasis in vivo. *Cell Metab*. 2006; 3:449–461. [PubMed: 16753580]
44. Rajagopal S, Fang H, Patanavanich S, Sando JJ, Kamatchi GL. Protein kinase C isozyme-specific potentiation of expressed $\text{Cav}2.3$ currents by acetyl- β -methylcholine and phorbol-12-myristate, 13-acetate. *Brain Res*. 2008; 1210:1–10. [PubMed: 18420182]
45. Hell JW, Westenbroek RE, Warner C, Ahljianian MK, Prystay W, Gilbert MM, Snutch TP, Catterall WA. Identification and differential subcellular localization of the neuronal class C and class D L-type calcium channel $\alpha 1$ subunits. *J Cell Biol*. 1993; 123:949–962. [PubMed: 8227151]
46. Yokoyama CT, Westenbroek RE, Hell JW, Soong TW, Snutch TP, Catterall WA. Biochemical properties and subcellular distribution of the neuronal class E calcium channel $\alpha 1$ subunit. *J Neurosci*. 1995; 15:6419–6432. [PubMed: 7472405]
47. Stea A, Soong TW, Snutch TP. Determinants of PKC-dependent modulation of a family of neuronal calcium channels. *Neuron*. 1995; 15:929–940. [PubMed: 7576641]
48. Kamatchi GL, Tiwari S, Chan C, Chen D, Do SH, Durieux M, Lynch C III. Distinct regulation of expressed calcium channel 2.3 in *Xenopus* oocytes by direct or indirect activation of protein kinase C. *Brain Res*. 2003; 968:227–237. [PubMed: 12663092]
49. Jeffrey D, Dula S, Corbin K, Wu R, Nunemaker C. A practical guide to rodent islet isolation and assessment. *Biol Proced Online*. 2009; 11:3–31. [PubMed: 19957062]

Fig. 1.

Western blot (1 A) showing the down regulation of Ca_vβ subunits and the blood glucose and serum insulin levels (1 B) after the administration of Ca_vβ subunit siRNAs. Western blotting and GTT were conducted 24 hours after the administration of the siRNAs. Pancreatic islet protein extracts (100 μg/lane) from the scrambled or Ca_vβ siRNA injected mice were used for the Western blots. Specific antibodies for Ca_vβ1 - β4 subunits and GAPDH were used for test and control respectively. GTT was conducted after 12 hours of food deprivation and with i.p., glucose challenge. ^d<0.05, ^c<0.02, ^a<0.001 compared to respective scrambled siRNA using t-test; n = 4–7. CF, cytoplasmic fraction; MF, membrane fraction; scr., scrambled.

Fig. 2.

Insulin determination in intact pancreatic islets (A) and in cultured pancreatic islet cells (B) after challenging with two concentrations [3 mM (basal) and 16.7 mM] of glucose. A. Three equal-sized islets each from the scrambled or $Ca_v\beta$ siRNA injected mice were used in this study. B. Cultured pancreatic islet cells from naïve mice were transfected with scrambled or $Ca_v\beta$ siRNA as described under the methods. These cultures were challenged with 3 mM or 16.7 mM glucose and insulin release was determined after one hour. $d < 0.05$, $b < 0.01$, $a < 0.001$ compared to respective scrambled siRNA using t-test; $n = 3$. scr., scrambled.

Fig. 3.

Confocal microscopy of cultured pancreatic islet cells showing translocation of PKC isozymes. Only the isozymes that were translocated by Ca $_v\beta$ siRNA are shown in the figure. Cultured pancreatic islet cells from naïve mice were cotransfected with scrambled or Ca $_v\beta$ 2 or β 3 siRNA (2.5 μ g each) and selected PKC isozyme cDNA (2.5 μ g each) subcloned into GFP containing vector. The translocation of the PKC isozymes was studied by immunocytochemistry as described under the methods. Bar, 10 μ M. scr., scrambled.

Fig. 4.

Blood glucose (top panel) and serum insulin levels (bottom panel) after the administration of PKC isozyme siRNA. GTT was conducted 24 hours after the administration of the siRNAs. The GTT was conducted with i.p., glucose challenge in mice deprived of food for 12 h. ^d<0.05, ^b<0.01 compared to respective scrambled siRNA using t-test; n = 3. scr., scrambled.

Fig. 5.

Confocal microscopy of cultured pancreatic islet cells showing the down regulation of PKC isoforms. Cultured pancreatic islet cells from the naive mice were cotransfected with scrambled or PKC α , β II or θ siRNA and PKC α , γ or ϵ cDNA (top panel), PKC β II, γ or θ cDNA (middle panel) and PKC θ , ϵ or β II cDNA (bottom panel). The cDNA for PKC isoforms are subcloned into GFP containing vector. 2.5 μ g each of PKC isoform siRNA and PKC isoform cDNA were used for the cotransfection. The cotransfection and immunohistochemistry were conducted as described under the methods. Bar, 10 μ M. scr., scrambled.