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Regulation of insulin exocytosis by calcium-dependent protein kinase C in beta cells

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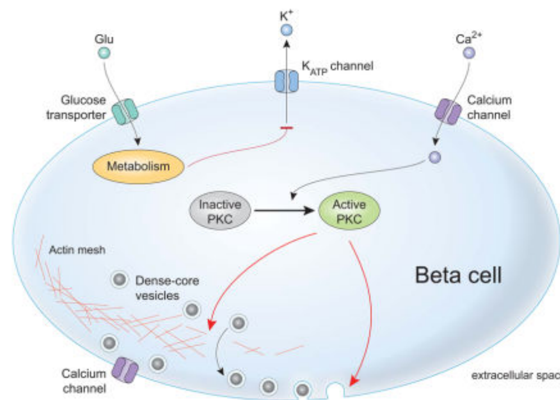
Abstract

The control of insulin release from pancreatic beta cells helps ensure proper blood glucose levels, which is critical for human health. Protein kinase C has been shown to be one key control mechanism for this process. After glucose stimulation, calcium influx into beta cells triggers exocytosis of insulin-containing dense-core granules and activates protein kinase C via calcium-dependent phospholipase C-mediated generation of diacylglycerol. Activated protein kinase C potentiates insulin release by enhancing the calcium sensitivity of exocytosis, likely by affecting two main pathways that could be linked: 1) the reorganization of the cortical actin network, and 2) the direct phosphorylation of critical exocytotic proteins such as munc18, SNAP25, and synaptotagmin. Here, we review what is currently known about the molecular mechanisms of protein kinase C action on each of these pathways and how these effects relate to the control of insulin release by exocytosis. We identify remaining challenges in the field and suggest how these challenges might be addressed to advance our understanding of the regulation of insulin release in health and disease.

Graphical Abstract



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Exocytosis and insulin release

Mammalian physiology demands exquisite control over insulin release and blood glucose levels. The debilitating and potentially fatal consequences of dysfunction in the insulin release system are seen in diseases such as diabetes. Careful physiological regulation would be impossible if an endocrine cell's entire store of insulin was released upon an initial stimulus, rendering the cell unable to respond to subsequent challenges. Instead, there are multiple levels of control to ensure a sustained and balanced release of insulin from beta cells of the pancreas. This review focuses on protein phosphorylation by protein kinase C (PKC), one primary regulatory mechanism which acts at multiple steps in the complex signaling cascade controlling insulin secretion.

Over fifty years of study of pancreatic beta cells have led to a comprehensive understanding of how glucose initiates the signaling cascade that leads to insulin secretion [1–4] (Figure 1A). At a simplified level, when blood glucose is elevated, transporters move glucose into beta cells of the pancreatic islets. As these sugars are metabolized, the cytoplasmic ratio of ATP/ADP increases, closing ATP-sensitive potassium channels (K-ATP channels) and blocking potassium efflux from the cell [5]. The resulting depolarization of the cell's plasma membrane opens voltage-gated calcium channels, allowing extracellular calcium to enter the cytoplasm (Figure 1A). Like many regulated secretory systems, elevated intracellular calcium is the ultimate trigger for exocytosis, stimulating the fusion of insulin-containing dense-core granules with the plasma membrane and the release of their luminal contents into the blood [6]. With glucose stimulation, beta cells exhibit a biphasic pattern of insulin release composed of an intense and transient first phase followed by a second sustained phase that builds slowly over time [2, 5]. The pathway described above is active in both phases of biphasic secretion and oscillatory changes in calcium underlie the two-component behavior. In addition to electrogenic effects, glucose metabolism potentiates release in pathways that act downstream of calcium influx. Additionally, other pathways, such as receptor-mediated cAMP elevation contribute to the control of calcium signaling and may directly activate protein kinases [7]. These other pathways integrate extracellular signals from neurotransmitters or hormones and provide additional layers of control over insulin secretion often via cAMP-dependent pathways [7, 8]. In the end, all these signals funnel into the same conserved molecular machinery that drives the fusion of insulin-loaded granules with the plasma membrane.

In beta cells the fusion of exocytotic vesicles is driven by a macromolecular complex composed of over a dozen proteins and lipids [6] (Figure 1B). This machinery is broadly conserved in regulated exocytosis from yeast to humans and across diverse secretory systems that are found in endocrine, neuroendocrine, neuronal, cardiac, reproductive and other tissues. The core components are the SNARE proteins: syntaxin, SNAP25, and VAMP [6]. These three proteins are embedded in opposing vesicular (VAMP) and plasma membranes (syntaxin and SNAP25) and interact to form a four-helical bundle when secretory vesicles dock at the plasma membrane. When exocytosis is initiated, the helical bundle zippers completely, which produces mechanical force that pulls the two membranes into close apposition. *In vitro*, the SNAREs are sufficient to drive membrane fusion [9, 10]. However, in cells additional proteins control the distinct steps in a secretory vesicle's

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lifecycle: 1) translocation to and docking with the plasma membrane, 2) priming that activates the vesicle to fuse, and 3) fusion pore formation (Figure 1B). For example, secretory vesicles are coated in small Rab GTPases such as Rab27a and Rab3a, and their effectors, including rabphilin and granuphilin [11]. These proteins play key roles in vesicle trafficking and docking. Priming is believed to involve partial assembly of the SNARE proteins mediated by several modulators including munc13, munc18, CAPS, tomosyn and others [6]. Finally, membrane fusion needs the primary calcium sensor for exocytosis, synaptotagmin, a vesicular membrane protein, which binds calcium and helps to catalyze membrane fusion through still-controversial mechanisms [12–14]. Together these molecules compose the macromolecular assembly that mediates vesicle fusion in a carefully regulated manner. One pathway for regulation of this process is PKC [15].

PKC potentiates insulin release in beta cells; however, it is currently not clear how this is mechanistically accomplished in living cells [15]. First, we discuss PKC structure, regulation, and activation in beta cells. Then, we address two fundamental questions: 1) what is the specific effect of PKC on insulin release, and 2) how does PKC achieve these effects? In the context of these questions, we discuss the current models for PKC action on glucose-stimulated insulin release and further examine three exocytotic proteins that are likely targets of PKC and control vesicle fusion at the plasma membrane.

PKC structure and activation

PKC isozymes are ubiquitous serine-threonine kinases with importance to human health and physiology, as evidenced by their role in many diseases including heart disease, cancer, and diabetes [16]. PKC isozymes are highly regulated; the conventional isozymes require the regulatory lipid diacylglycerol (DAG), negatively charged phospholipids phosphatidylserine and phosphatidylinositide-4,5-bisphosphate (PIP2), and calcium [17]. PKC activity was initially identified in pancreatic islets by Tanigawa et al., and this activity arises from expression of many PKC isoforms in beta cells, including α, βII, ε, λ, and ζ and possibly βI, θ, and η [18, 19].

In beta cells, PKC is activated during glucose stimulated insulin secretion (GSIS) when DAG is produced during calcium influx [20]. DAG production occurs when glucose-induced calcium influx first activates phospholipase C (PLC), which hydrolyzes some of the PIP2 in the membrane to yield DAG and inositol triphosphate (IP3) [21]. In addition to calcium, many PLC isoforms are activated via G-protein coupled receptor pathways, some of which contribute to the regulation of insulin secretion and secretion amplification [22, 23]. Glucose alone can induce both DAG production in islets and subsequent PKC translocation to the plasma membrane, a hallmark of enzyme activation [20, 24–26]. These findings mechanistically link PKC activation to the physiological stimulus that activates beta cells.

There are eight PKC isozymes divided into three classes based on the molecules that activate them [17]. These are 1) conventional PKCs, activated by calcium and DAG; 2) novel PKCs, activated by DAG alone; and 3) the atypical PKCs, activated by neither calcium nor DAG [17]. At the structural level, PKCs have two common domains: the kinase domain, composed of an ATP dependent active site and region for binding the substrate consensus

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sequence, and the regulatory domain, composed of conserved C1 (binds DAG molecules) and C2 (binds PIP2 via calcium-dependent mechanism) and pseudo-substrate domains [17] (Figure 2A). In its inactive state, the regulatory domain auto-inactivates the kinase domain by positioning the pseudo-substrate sequence in the active site of the kinase domain (Figure 2C-1). Recent structural work on PKC-β suggests a second mechanism of allosteric regulation whereby the C1 domains clamp a critical helix in the kinase domain, which must be released for kinase activation [27, 28] (Figure 2B). In the presence of calcium, the C2 domain binds PIP2 and PS headgroups, tethering the enzyme to the plasma membrane (Figure 2C-2). After membrane association, the C1 domains can interact with DAG. DAG binding then activates the kinase by inducing a conformational change which removes the pseudo-substrate from the active site and releases the C1 clamp. (Figure 2C-3) For conventional PKC, tripartite regulatory interactions provide spatiotemporal control over enzyme activity in the cell by restricting PKC activation to only organelles and regions that simultaneously contain all three of these regulatory modules.

What is the effect of PKC on insulin release?

For close to 40 years PKC has been known to potentiate insulin release from beta cells [29, 30]. The foundational work on PKC's effects on exocytosis relied on the application of phorbol esters (phorbol 12-myristate 13-acetate (PMA or also abbreviated TPA)). Phorbol esters act as analogs to the lipid DAG that, together with calcium, activate conventional PKCs [31–33]. Consequently, enhanced insulin release upon PMA treatment was taken as evidence that PKC stimulates insulin secretion [34–37]. The use of PKC inhibitors supported this conclusion [38–41]. Additionally, in several cell types, the application of exogenous PKC to permeabilized cells enhanced exocytosis, providing further evidence for a direct effect of the enzyme on secretion [42, 43]. PKC activation and translocation to the membrane are linked, and thus in addition to correlating enhanced secretion with PMA, the necessary translocation of PKC to the plasma membrane upon PMA treatment has also been observed [24, 25, 38, 44–49]. These experiments first took the form of enzymatic assays of PKC activity that was associated with the plasma membrane [38]. Further biochemical and imaging studies showed that PKC isozymes α, β, ε, and θ all translocate to the plasma membrane or insulin granules upon activation with several compounds (glucose, PMA, or potassium-driven depolarization) [24, 25, 44–49]. Additionally, there is evidence that atypical PKC isozymes may play a role in secretion [50]. Overall, there is strong consensus that PKC activation enhances insulin release.

PKC does not, however, directly stimulate secretion by initiating calcium influx into the cell [51]. Work from multiple groups measuring calcium currents has shown that PMA alone or coupled with glucose does not modulate intracellular calcium influx [34, 51–53]. Instead of contributing to calcium influx, the application of PMA shifts the calcium sensitivity of exocytosis to lower calcium concentrations. This was originally shown in chromaffin cells [54, 55] and then in beta cells [56–59] using a variety of cracked or patched cell systems where the calcium concentration can be clamped. In pioneering work from Zawalich et al. (1983), it was shown that the combination of a calcium ionophore and PMA could reproduce the classic biphasic insulin release pattern [36]. Calcium ionophores produced the initial spike in insulin release, and PMA treatment supported the second phase of insulin release in

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the continued presence of the ionophore. Indeed, PKC activation alone is not required for the first phase of insulin secretion after glucose stimulus [60, 61]. Together, these early studies suggested PKC potentiates insulin released in response to glucose stimulation, but PKC itself cannot initiate release [62]. PKC likely increases the calcium sensitivity of the exocytotic machinery, thereby potentiating the overall amount of insulin secreted in response to a given calcium stimulus.

Aside from studies in model systems of insulin secretion, there is evidence that PKC dysfunction could play a role in diabetes. Aberrant PKC activity, perhaps through misregulation of PKC isozymes, might underlie the diabetic phenotype in Goto-Kakizaki rats, a common animal model for type 2 diabetes [63–65]. Furthermore, PKC-ε knockout in a mouse model prevented glucose intolerance and inhibition of PKC-ε increased insulin secretion in diabetic mice, suggesting a role of this isozyme in disease [66]. In new work, the immediately-releasable pool of vesicles, composed of vesicles tightly associated with calcium channels, was observed to be absent in insulin-secreting cells from human donors with type II diabetes [67]. As discussed below, interaction between L-type calcium channels and insulin granules may be modulated by PKC.

A word of caution before we consider how PKC enhances insulin release. Although a useful pharmacologic tool, the broad activity of phorbol esters likely has contributed to significant confusion in the field. It was noted at least as early as 1985 by Tamagawa et al. that other targets of PMA could be contributing to the drug's effects on insulin release [59, 68]. For example, munc13, an essential factor in vesicle priming and exocytosis, is a phorbol ester receptor [69, 70]. Studies have identified munc13 as being crucial for the second phase of insulin release, the phase in which PKC was originally postulated to act [36, 71, 72]. Though more recent work in neurons has developed an integrated model in which both PKC-dependent (possibly via munc18) and PKC-independent (possibly via munc13) pathways are necessary for exocytosis [73, 74], the wide-ranging targets of PMA make deconvoluting the specific effects of PKC challenging.

How does PKC globally enhance insulin release?

Consistent with its ubiquitous activity, PKC likely acts on many targets to regulate insulin release. Here, we distinguish between global cell-wide PKC effects (Figure 1A) and local effects on protein targets directly involved in exocytosis of dense core vesicles (Figure 1B). First, we will cover PKC modulation at global sites:

1. PKC could regulate proteins that control the structure and dynamics of the cortical actin network and thus allow more insulin granules to dock at the plasma membrane.

2. PKC's effect on calcium channels has been controversial, but a direct means to regulate insulin granule exocytosis could be to directly modulate calcium channel activity or location.

3. PKC helps to control many global cellular processes including transcription and cell metabolism. Because insulin secretion is linked to cellular ATP levels, effects on cell metabolism could indirectly affect exocytosis.

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One global effect of PKC activation is actin rearrangements that allow vesicles to move towards the plasma membrane. Many secretory cells have a ~100 nm-thick dense cortical network of actin near the plasma membrane that is thought to act as a physical barrier to vesicles [75]. PKC-induced reorganization of this actin layer has been observed in multiple systems: beta cells, chromaffin cells, neuronal systems, and others [47, 53, 76–79]. Rearrangement of the actin cortex has been interpreted as having two effects. First vesicle pools near the plasma membrane are larger (in the case of pre-stimulus PKC activation), which accounts for increased initial rates of exocytosis from the readily-releasable pool (RRP) [53, 80]. The second effect is to enhance RRP refilling after initial stimulation to potentiate sustained release or cell adaptation [53, 81]. The sustained phase of insulin release must include the recruitment of additional insulin granules from the reserve pool to the plasma membrane, consequently it seems likely PKC-mediated cortical actin rearrangement could play a prominent role in potentiating sustained insulin release.

One PKC target that controls cortical actin structure is a well-known actin crosslinking protein, myristoylated alanine-rich C-kinase substrate (MARCKS). MARCKS cross-links actin and binds PIP2, which is regulated by PKC phosphorylation [82]. Activation of PKC releases MARCKS from the plasma membrane and has been directly correlated with cortical actin disassembly in chromaffin cells [47, 76]. In insulin-secreting cells, the translocation of PKC to the plasma membrane and release of MARCKS has been directly visualized [46]. These studies demonstrate that a global effect of PKC activation is the re-organization of the cortical actin cytoskeleton through loss of MARCKS from the plasma membrane.

In addition to MARCKS, several other proteins directly modified by PKC are likely playing a role in actin rearrangement and vesicle movement. Vesicle-localized myosin may be regulated via PKC, perhaps via myosin light chain kinase (MLCK), which is a PKC target [83]. 14-3-3 protein was originally identified as playing a PKC-dependent role in exocytosis as well [42, 84]. Subsequent work has shown 14-3-3 likely acts as a scaffolding factor between vesicle-bound Rab-effector proteins and other molecules, such as myosin, however, it may be dependent on other kinase systems [85, 86].

Another possible target of PKC is the plasma membrane calcium channels necessary for insulin release. Modulation of these channels could provide a direct mechanism to increase the calcium response to glucose metabolism and consequently increase the exocytotic response. Beta cells have L-type, R-type, and P/Q-type calcium channels [87]. L-type channels are the primary drivers, though are not required, for GSIS [87–90]. Early reports indicated PKC activation could cause calcium influx and inhibition of PKC could block exocytosis [33, 91–93], however, these effects may have been caused by intracellular calcium stores [93, 94]. The current weight of evidence shows no change of intracellular calcium levels, or indeed inhibition of calcium currents, upon PKC activation leading to the conclusion that PKC does not directly modulate calcium levels in the cell [34, 51–53, 59, 95]. Additional confusion likely also arose due to phorbol ester PKC-independent effects on channel behavior [96, 97]. PKC’s primary effect is to enhance the calcium sensitivity of exocytosis rather than to amplify or modulate calcium currents via channel activity.

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To the extent that PKC may influence calcium channels, it could regulate interactions between calcium channels and binding partners, which could affect exocytosis without causing large changes in calcium currents. Yokohama et al. demonstrated that Cav2.2 channels are direct PKC targets, and that phosphorylation modulates interaction of syntaxin with the channel [98]. Indeed, deletion of the syntaxin interaction site on these channels or expression of a peptide competitor for the interaction site blocks exocytosis, pointing to an important role for PKC's modulation of this interaction [99, 100]. If syntaxin interaction with Cav2.2 primarily serves to localize the channel to the exocytotic machinery [101], rather than modulating channel activity, this may reconcile the findings that PKC does not affect calcium channel currents but that channel modification by PKC is an important regulatory mechanism. Proximity to calcium channels would provide syntaxin and the exocytotic machinery with high local calcium concentration, which is hypothesized to be a mechanism for generating the immediately-releasable pool of exocytotic vesicles [102]. Importantly, it was recently found that this pool of vesicles is absent in cells from human donors with type II diabetes [67], which strongly suggests that modulation of the L-type channel-syntaxin interaction by PKC could be critically important in diabetes.

Finally, PKC can readily affect cell metabolism, which has an indirect impact on insulin secretion [103]. Several studies have shown some PKC isoforms target transcription factors [24, 104, 105]. These factors regulate the expression of metabolic genes, such as hexokinase, and consequently influence calcium currents and insulin release along the standard GSIS pathway by affecting ATP levels in the cell [22]. For example, generation of a PKC-ε knockout-mouse showed that this specific PKC likely plays a role in regulating metabolic pathways in beta cells [66]. Insulin protein synthesis is not necessary for continued insulin secretion from islets for several hours [2, 4], so it is unlikely that any PKC effects on protein synthesis would be relevant to insulin release over this timescale. Overall, the global effects of PKC regulation on cell metabolism must be very complex and more work is needed to understand PKC's contribution to cellular ATP levels.

What are the local exocytotic protein targets of PKC?

A direct method for PKC to potentiate insulin release would be to phosphorylate and activate components of the exocytotic machinery. Bulk measurements show that potentiation occurs by enhancement of the calcium sensitivity of exocytosis [56–59]. At a basic level this results in more insulin released per calcium stimulus. Several possible pathways could work to achieve this:

1. PKC could directly alter the ability of single vesicles to fuse by modifying proteins involved in vesicle priming and fusion, thus increasing the total number of exocytotic events per cell [15, 78].

2. PKC could enhance vesicle docking, by modifying proteins involved in docking interactions, to increase the size of the docked vesicle pool. More docked vesicles with the same probability of fusion per vesicle could produce an enhancement of total insulin release [52].

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3. PKC could modulate the amount of insulin cargo released from single vesicles in kiss-and-run type exocytotic events [106]. Far less work has been done to directly study this phenomenon, and kiss-and-run exocytosis appears to be a minor pathway in beta cells [107].

Here, we discuss several exocytotic protein targets that could mediate these possible mechanisms.

Many exocytotic proteins are targets of post-translational modification by a host of kinases [15, 108–110]. *In vitro*, PKC phosphorylates syntaxin [108, 110]. Also, PKC can phosphorylate VAMP [108, 111], though other kinases are likely to be more relevant [15]. NSF is a PKC target and phosphorylation appears to reduce its affinity for SNARE complexes [112]. There is some evidence in neurons that NSF and PKC-ε control trafficking of receptors to the cell surface [113]. However, although many of these proteins can be phosphorylated *in vitro*, there remains little direct evidence for these phosphorylation events playing a dominant role in insulin secretion. In contrast, the most well studied PKC targets in the exocytotic machinery are munc18, synaptotagmin, and SNAP25 [15].

Munc18 is a syntaxin-binding protein that plays a critical docking and priming role in exocytosis [114] (Figure 1B1-3). Munc18 binds to the closed form of syntaxin, but also interacts with the ternary SNARE complex via a binding site on syntaxin's N-terminus [114]. Munc18 is phosphorylated by PKC at residues Ser306 and Ser313 [115, 116]. *In vivo* munc18 phosphorylation has been observed in many cell systems: islets, chromaffin cells, neurons, and others [117–122]. In neuronal synapses, munc18 phosphorylation is kept low by phosphatases, such that upon PKC activation a large dynamic change in phosphorylation state can be achieved [118, 123]. Munc18 appears to be important in RRP refilling, which could be attributed to a syntaxin trafficking role or to an increase in SNARE ternary complex assembly [119, 120]. Overall there is good evidence that munc18 is regulated by PKC *in vivo* and that the protein's phosphorylation state is dynamically controlled, suggesting it could be an important regulatory module.

How does munc18 phosphorylation relate to insulin release? A longstanding hypothesis is that phosphorylation of munc18 causes opening of a closed form of syntaxin and allows for more vesicles to dock to the plasma membrane [115]. This would increase the number of calcium-sensitive vesicles at the plasma membrane. Another hypothesis comes from work by Mandic et al. who looked more closely at munc18-1 and munc18-2, two isoforms expressed in beta cells, and their function in insulin secretion [124]. Interestingly, these authors show that munc18-1 and munc18-2 support exocytosis with distinct kinetics and that the two isoforms affect the calcium sensitivity of vesicles at the membrane. Phosphorylation of both is important for their function. Differential phosphorylation and activation of these isoforms could be the molecular correlate of the PKC-dependent calcium sensitivity changes of exocytosis in beta cells. Ultimately, the mechanistic details of munc18 phosphorylation with respect to insulin release remains unclear.

Synaptotagmin is the primary calcium sensor of exocytosis, making it an ideal candidate for PKC-mediated calcium sensitization of the exocytotic apparatus [125] (Figure 1B3). However, phosphorylation of synaptotagmin does not appear to directly affect its calcium

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affinity. The protein is phosphorylated by PKC at Thr112, and phosphorylation levels increase when PKC is activated by PMA [126]. There are many synaptotagmin isoforms, with Syt-1 and Syt-7 playing major roles in neuronal and endocrine secretion, respectively [125]. Synaptotagmin isoforms have different affinities for calcium, suggesting that they could mediate exocytosis of distinct vesicle pools *in vivo* [125]. Sorting of Syt-1 and Syt-7 onto distinct vesicle pools occurs in chromaffin cells and affects exocytotic behavior [127].

Evidence suggests that phosphorylation increases synaptotagmin affinity for the SNARE complex [15, 128]. It is, however, unclear how this might affect release. Synaptotagmin has been identified as part of the minimal vesicle docking machinery in chromaffin cells [129]. Thus, phosphorylation of synaptotagmin could modulate docking and because vesicle fusogenicity is heterogeneous, docking of different vesicle populations could lead to changes in apparent calcium sensitivity of exocytosis. For example, phosphorylation of synaptotagmin-7 by PKC would enhance its affinity for the SNARE complex and consequently enhance docking of synaptotagmin-7-labeled vesicles with the plasma membrane. This could lead to a change in the apparent calcium sensitivity of exocytosis, as synaptotagmin-7 has a higher calcium affinity than synaptotagmin-1 [127]. Consistent with a scenario under which different synaptotagmin isoforms are relevant PKC targets in different cellular systems, Nagy et al. showed in chromaffin cells that overexpression of synaptotagmin-1 phosphomutants and phosphomimetics did not appear to alter exocytosis, whereas in another system, hippocampal neurons, phosphomimetics of synaptotagmin-1 regulate exocytosis [73, 130]. Synaptotagmin isoform-specific phosphorylation has yet to be demonstrated, but it represents an exciting hypothesis for the molecular mechanisms underlying the sensitization of the exocytotic machinery by PKC.

Finally, SNAP-25 appears to be a PKC substrate *in vivo*, though the role of this modification in insulin secretion is still controversial. The most compelling evidence for physiologically relevant phosphorylation-dependent control of SNAP-25 is that knock-in mice expressing a SNAP-25 mutant that cannot be phosphorylated display neurological defects including anxiety and epilepsy [131, 132]. SNAP-25 phosphorylation was first observed *in vitro* and was quickly shown to occur in response to PMA treatment in PC12 cells, correlating with enhanced exocytosis [109, 133]. The primary site for PKC-mediated phosphorylation of SNAP-25 is Ser187 [133]. Further studies in insulin-secreting, neuroendocrine, and neuronal systems have been at odds over whether PKC-mediated phosphorylation of SNAP25 directly affects or is only correlated with exocytosis [134–139]. It is currently thought that phosphorylation of SNAP-25 increases its interaction with syntaxin and that this increases the population of the highly-calcium sensitive pool of vesicles [139, 140]. More recent work suggests that endogenous SNAP-25 may have complicated earlier studies on phosphomimetic SNAP-25 mutants [138, 139]. Despite phenotypic effects in neurons of model organisms, SNAP-25's physiological role in insulin secretion remains controversial.

In addition to the exocytotic proteins discussed above, PKC could affect insulin release by modulating the amount of cargo released at single vesicles (Figure 1B3-4). More complete release of insulin from single vesicles could enhance the overall amount of insulin released without changing the total number of fusion events per cell. Early evidence for a role for PKC modulating fusion pore behavior in neuromuscular junctions came from the

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observation that staurosporine, a PKC inhibitor, affected fusion pore behavior and vesicle collapse into the plasma membrane [141]. In neurons, PKC was shown to affect exocytosis after calcium binding to synaptotagmin, suggesting that fusion pore regulation could play a role [142]. In chromaffin cells, amperometry has been used to examine the kinetics of the fusion pore at very high time resolution. PKC activation appears to accelerate fusion pore expansion [117, 143, 144], however, some studies have also shown that PKC tends to decrease the average quantal size of fusion events [117, 143]. Quantal size could be linked to the probability of kiss-and-run exocytosis, which may be a function of the stimulation protocol, making it challenging to interpret these findings with respect to physiological glucose stimulation.

PKC's effect on quantal size may involve regulation of endocytotic protein components responsible for mediating kiss-and-run exocytosis. There is a growing body of evidence to suggest that canonical endocytotic proteins—dynamin, amphiphysin, syndapin, and others—may play a role in modulating fusion pore expansion or collapse via a kiss-and-run mechanism [127, 145–149]. Dynamin and other endocytotic proteins are known to be modulated by PKC activity [150, 151]. By targeting these proteins, PKC could be modulating fusion pore dynamics or closure and thus affecting the amount of insulin release. This hypothesis has not been fully explored and future work with insulin granules are an ideal system to determine if kiss-and-run or sub-quantal cargo release could play an important role in physiological insulin levels.

PKC may also modulate fusion pore expansion and kinetics through its control of exocytotic protein factors. Barclay et al. suggest munc18 phosphorylation has a direct effect on fusion pore kinetics, though munc18 itself is unlikely to have direct effects on the membrane [117]. Instead munc18 could be either allowing more trans-SNARE complex assembly or enabling more efficient zippering of SNAREs [152, 153]. In addition to modulating the amount of SNARE complex formation, munc18 could directly affect syntaxin-mediated regulation of fusion pore diameter and behavior [154]. Munc18 also appears to help to regulate the selection of larger diameter vesicles for docking and fusion, and vesicle size directly contributes to fusion pore size [154]. Recent work has implicated synaptotagmin in fusion pore kinetic control [127], though it is not clear how its phosphorylation might affect this putative function. Obviously, lipids play a central role in fusion pore structure and dynamics [155], and Xue et al. invoke a lipid-mediated mechanism to account for PKC effects on fusion pore dynamics [144]. PKC activates phospholipase D, an enzyme which produces phosphatidic acid via the cleavage of phosphatidylcholine in the membrane. Phosphatidic acid has a small headgroup and high spontaneous negative curvature that would help to accommodate the high membrane curvatures at the nascent fusion pore. Overall, it seems possible PKC could exert an effect at the nascent fusion pore itself, though the mechanism of this remains unclear and requires further study, particularly with respect to insulin granules.

Bringing it all together

At the organismal level, activation of PKC enhances the amount of insulin that islets release into the blood upon glucose challenge. Potentiation of exocytosis occurs primarily in the

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second sustained phase of insulin release. PKC likely mediates this process by acting on two pathways that are connected: increasing the size of the RRP and sensitizing the exocytotic apparatus to calcium.

The transient first phase of GSIS corresponds to fusion and depletion of the RRP, so refilling this pool is necessary for the second, sustained phase of GSIS. RRP refilling requires the partial disassembly or re-organization of the cortical actin network, which allows more insulin granules to translocate to the plasma membrane. PKC may regulate this step via phosphorylation of MARCKS, an actin-crosslinking protein directly implicated in actin disassembly.

PKC also potentiates insulin release by enhancing the calcium sensitivity of the exocytotic machinery, but it remains unclear how this is achieved. No known modifications of exocytotic proteins directly increases their calcium affinity, which would be the most straightforward mechanism to enhance the calcium sensitivity of exocytosis. One mechanism to achieve calcium sensitization could involve isoform specific phosphorylation of munc18 or synaptotagmin. As reserve vesicles translocate to the plasma membrane, PKC may help to determine which vesicles successfully dock. Different isoforms of both munc18 and synaptotagmin display distinct calcium affinities that could be used to modulate exocytotic calcium sensitivity [124, 125]. Such a model is consistent with observations that PKC acts to increase the size of the highly calcium-sensitive vesicle pool [156–158]. Several studies have shown newcomer vesicles are more likely to fuse and are more calcium sensitive, which is consistent with this model [159, 160]. Another possible mechanism to enhance the calcium sensitivity of exocytosis is to increase the size of the RRP [52]. Phosphorylation of all three of the exocytotic proteins we discussed—munc18, synaptotagmin, and SNAP25—could enhance vesicle docking by either providing more docking sites or increasing the affinity of the docking machinery for vesicles. A larger pool of docked vesicles with a homogeneous calcium sensitivity for each vesicle could yield a larger exocytotic response to a given calcium stimulus [52].

It is possible that both PKC effects on cortical actin as well as specific exocytotic proteins could contribute to a unified mechanism of enhance insulin release. Cortical actin disassembly enables vesicle translocation from the reserve pool and phosphorylation of exocytotic proteins with known roles in vesicle docking would then ensure that newly arrived vesicles dock with the plasma membrane. Both mechanisms contribute to enlarging the docked, potentially active vesicle pool and thus giving rise to a larger insulin release upon a calcium stimulus.

Challenges and open questions

Despite ongoing characterization of PKC modifications to exocytotic proteins, we still lack a unified understanding linking these changes to PKC’s effect on bulk insulin release. This is due in part to the complexity of GSIS. Insulin secretion is several steps removed from the introduction of glucose. Each step is regulated, and PKC could act at several of them. Other regulatory enzymes act in concert with PKC. Additionally, PKC isozymes act in different signaling pathways and the isolation of their individual effects on exocytosis is challenging.

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Compounding the difficulties in sorting out these pathways, past tools for modulating the activity of PKC necessary to gain mechanistic insight into their roles in GSIS have been relatively non-specific.

Deepening our understanding of PKC's role in GSIS will require harnessing recently developed techniques and developing new tools. Here we describe three challenges remaining in the field and offer suggestions on experimental approaches to address them.

1. Despite good evidence that munc18, synaptotagmin, and SNAP-25 are physiological PKC targets, we do not yet have a unified theory of their roles in specific cell types. In isolation, it appears that each protein plays a role in exocytotic regulation, but their combined effects have not been studied. This question is likely an arena where advances in quantitative proteomics coupled with crosslinking methods could provide clarity. With APEX2 or similar methods combined with phosphoproteomics, an organelle specific, quantitative examination of the modifications of munc18, synaptotagmin, and SNAP-25 could be performed from the same sample [161]. Another powerful technique to compare modifications of exocytotic proteins in living cells is the use of fluorescent biosensors for specific kinase activity on exocytotic proteins of interest [162]. The introduction of environmentally-sensitive fluorophores using mutagenesis on endogenous (transfection or CRISPR-based expression of labeling constructs of exocytotic proteins of interest) or exogenous (recombinant protein microinjected into cells) proteins could report on their phosphorylation state in real time in living cells after insulin secretion is stimulated.

2. In addition to the exocytotic proteins, we have described several cellular targets of PKC that are likely to be playing a role in GSIS potentiation. It is not currently clear if multiple PKC isozymes target these cellular locations, or if there is overlap in isozyme function. Specific pharmacologic activators and inhibitors of PKCs would first enable further unraveling of how individual isozymes might have specific roles to play in GSIS. To some extent, specific peptide-based inhibitors have been used in the past, and more isoform-specific activator and inhibitors would be illuminating. Additionally, though distinct isozyme translocation in time and space has been observed, it is not clear where exactly PKC is being localized in each case. Multiple sites-of-action—the actin cortex, vesicles, and the plasma membrane—are all found within the same diffraction limited space. Super-resolution fluorescence microscopy or electron microscopy could resolve isozyme localization between these sites.

3. Lastly, we suggest that different isoforms of synaptotagmin could be differentially regulated by PKC which would provide the cell a means to control the calcium sensitivity of exocytosis. It would be of great interest to investigate if these different vesicle populations are subject to PKC regulation and respond uniquely to PKC activation. Targeted phosphoproteomics and live cell imaging could be used to identify which isoforms of synaptotagmin reside on vesicles at the membrane and which vesicles are most fusogenic upon stimulation.

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PKC plays an important role in modulating insulin secretion from pancreatic beta cells. Dysfunctional PKC signaling may be one mechanistic cause of diabetic phenotypes in model organisms for the disease, which underscores the importance of fully understanding PKC's contribution to the complex regulation of insulin release. PKC appears to act in multiple steps of GSIS, importantly both at the cortical actin network and at the level of individual exocytotic proteins that play key roles in mediating membrane fusion. With advances in targeted phospho-proteomics and live-cell super resolution fluorescence microscopy, we can move towards a unified model for how PKC acts at multiple sites *in vivo* to potentiate insulin secretion. Future work on PKC could lead to novel therapies or drugs to target exocytosis in the hopes of curing diabetes and other endocrine diseases.

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List of abbreviations

PKC	Protein kinase C
DAG	diacylglycerol
PIP2	phosphatidylinositol-4,5-bisphosphate
GSIS	glucose stimulated insulin secretion
PLC	phospholipase C
IP3	inositol triphosphate
PMA	phorbol 12-myristate 13-acetate
TPA	tetradecanoylphorbol acetate (synonym of PMA)
RRP	readily-releaseable pool
MARCKS	myristoylated alanine-rich C-kinase substrate
MLCK	myosin light chain kinase
DCV	dense-core vesicle

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Highlights

- Activation of protein kinase C potentiates insulin secretion from beta cells.
- Glucose stimulation produces calcium influx that activates conventional protein kinase C.
- Protein kinase C regulates cortical actin rearrangement that facilitates insulin release.
- Munc18, SNAP25, and synaptotagmin are exocytotic proteins likely regulated by protein kinase C.

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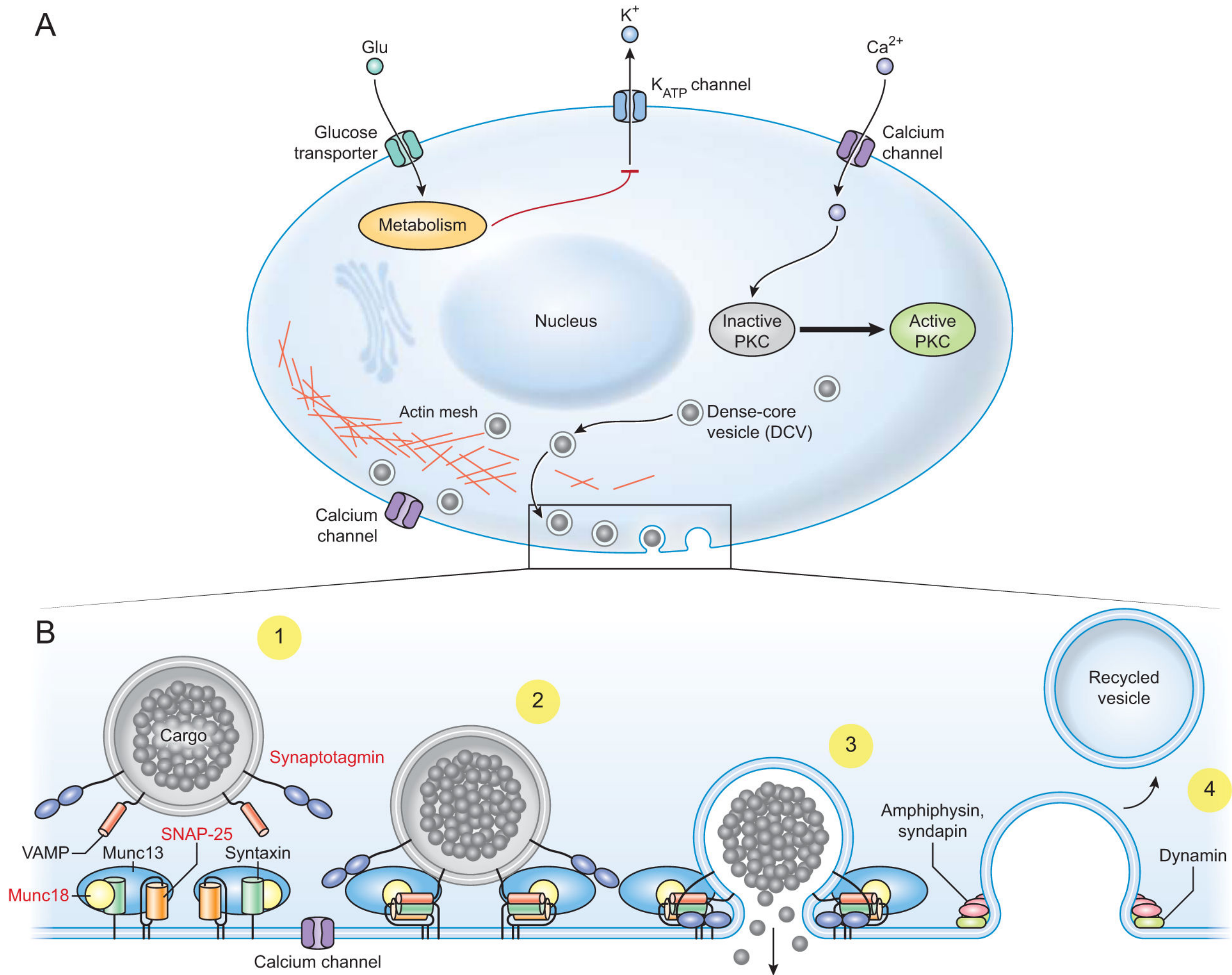

Figure 1.

The cellular and molecular pathways of glucose-stimulated insulin secretion. A) Glucose enters the cell and is metabolized, which alters the ATP/ADP ratio and closes K-ATP channels. The cell is depolarized which causes calcium influx via voltage-gated calcium channels and activation of PKC. PKC activation is shown schematically here but depends on calcium-mediated activation of phospholipase C, which produces the regulatory lipid diacylglycerol that acts on PKC. One major global function of PKC is to mediate reorganization of the actin cortex (shown bottom center of cell), which allows DCVs to move to the plasma membrane. B) The major molecular components of the exocytotic machinery are shown in schematic form in each stage of the exocytotic cascade. The principle exocytotic protein targets of PKC are highlighted in red in frame 1. 1) DCV translocation to the plasma membrane. 2) DCV docking and priming involves trans-SNARE complex formation along with co-factors like munc13, munc18, and synaptotagmin. 3) DCV fusion occurs upon calcium influx allowing insulin release. 4) Recycling can occur in kiss-and-run or cavicapture exocytosis where dynamin and other canonical endocytotic proteins close the fusion pore to recapture DCV membrane material. This can occur without full cargo release to modulate the amount of cargo released per exocytotic event.

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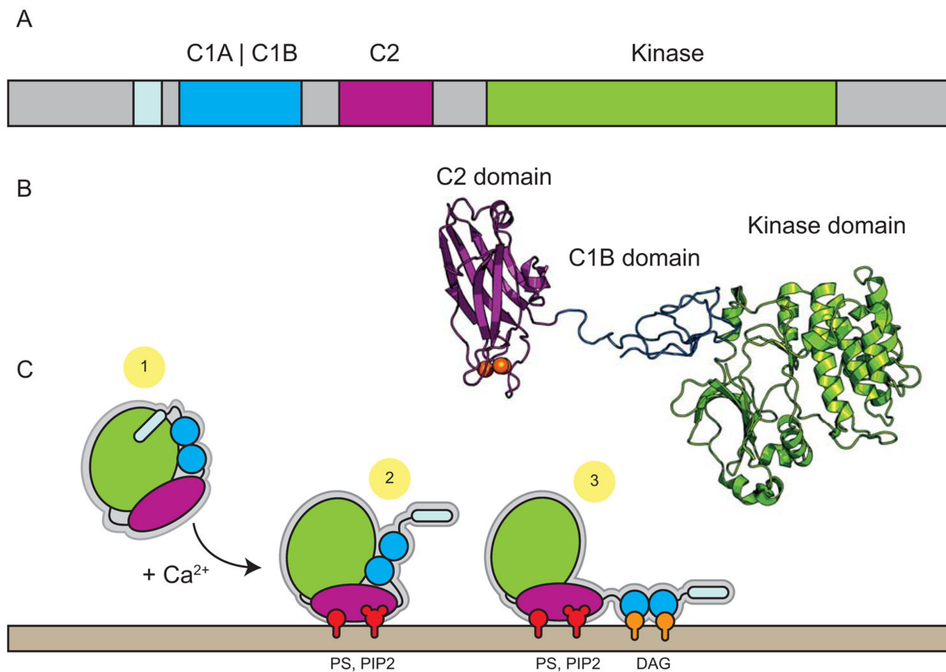

Figure 2.

The structure and activation of conventional PKC. A) The domain organization of conventional PKCs is roughly divided into the kinase domain (chartreuse) and the regulatory domain containing C1 (blue) and C2 (magenta) domains along with the pseudo-substrate domain (light blue). B) The crystal structure of PKCβ-II in its auto-inhibited form, with the C1B domain making contacts with the kinase domain (the C1 clamp). The PS and C1A domains and several linker regions were not visible in the structure (PDB: 3PFQ). The authors note that the extended arrangement of the C2 domain distal to the kinase domain is likely to be an artifact of crystal packing [27]. C) The activation of conventional PKC by calcium and lipids. 1) Cytosolic PKC is auto-inhibited by pseudo-substrate domain binding to the active site of the kinase along with the C1 clamp engaged with a critical helix on the kinase. 2) Calcium binds the C2 domain which mediates its interaction with PS and PIP2 lipids (red) in the membrane. 3) After translocation to the membrane, the C1 domains bind DAG (orange) which releases the C1 clamp and fully disengages the pseudo-substrate from the active site, thereby activating the enzyme.