New Horizons in Cellular Regulation by Inositol Polyphosphates: Insights from the Pancreatic β-Cell

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Abstract—Studies of inositol polyphosphates in the pancreatic β-cell have led to an exciting synergism between new discoveries regarding their cellular roles and new insights into β-cell function. Because the loss or malfunction of the β-cell is central to diabetes, these studies open the possibility of new pharmacological interventions in a disease that has reached epidemic proportions worldwide. Using the β-cell as our prime but not exclusive example, we examine the inositol polyphosphates in three main groups: 1) inositol 1,4,5-trisphosphate and its influence on Ca²⁺ signaling, specifically in a cell in which cytoplasmic-free Ca²⁺ concentration is principally increased by plasma membrane standing voltage-gated Ca²⁺ channels; 2) higher inositol polyphosphates including a novel second messenger inositol 3,4,5,6-tetrakisphosphate and a regulatory role for inositol hexakisphosphate in β-cell Ca²⁺ homeostasis and exo- and endocytosis; and 3) inositol pyrophosphates and their role in β-cell exocytosis, together with the exciting possibility of being novel targets for therapy in diabetes. We conclude with some of the new perspectives that are likely to become apparent in the next few years.

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I. Introduction

Inositol is a cyclitol whose structure has enabled the construction of a large family of stereochemically unique molecules that are engaged in every conceivable area of cellular regulation (Irvine and Schell, 2001; Irvine, 2005) (Fig. 1). The regulated release of insulin from pancreatic β-cells is one important cellular process in which inositol compounds have been found to play a significant role (Barker et al., 2002, 2009a; Berggren and Barker, 2008; Tengholm and Gyldé, 2009; Yamazaki et al., 2010). Indeed, there has been an exciting synergistic effect between the growth of our understanding of β-cell regulation and the discovery of novel functions for inositol-based structures, particularly the inositol polyphosphates (Barker and Berggren, 1999; Berggren and Barker, 2008; Barker et al., 2009a). This advancement in knowledge has important implications, because the defects in β-cell regulation underlie diabetes, a disease that has reached epidemic proportions worldwide. This information also has ramifications for the development of novel pharmacological strategies to treat this disease. We will first introduce both the pancreatic β-cell and inositol polyphosphates in more detail before returning to the synergism between them.

A. The Pancreatic β-Cell

The pancreatic β-cell is an excitable neuroendocrine cell in which the critical role is to control blood glucose concentration via the regulated release of the peptide hormone insulin. It is important to look broadly when considering its regulation, because in addition to the conventional glucose-driven stimulus-secretion coupling, it is subject to regulation by autocrine, paracrine, neuronal, and systemic factors (Berggren et al., 1990). Therefore, there is now an increasing appreciation that the microenvironment of the pancreatic islet, in which the β-cell resides, is critical for its function (Misler, 2010).

Nonetheless, the central platform of β-cell stimulus-secretion coupling is the linking of increases in blood glucose to the release of insulin (Fig. 2). As blood glucose is increased, it is reflected by an increased glucose uptake via glucose transporters. This increased glucose flux drives an increase in the ATP:ADP ratio that leads to the closure of ATP-sensitive K⁺ (KATP) channels. This influx of Ca²⁺ drives the final stages of insulin exocytosis (Berggren et al., 1993). An important aside to make for later discussions is that both the KATP and Ca²⁺ channels exist in special microenvironments in which the former is associated with the ATP-generating steps of glycolysis (Dhar-Chowdhury et al., 2005; Jovanovic et al., 2005) and the latter is physically coupled to the exocytotic machinery (Yang et al., 1999). Thus, other regulatory proteins likely to interact with these channels may be found in these microdomains.

Because blood glucose changes under physiologic conditions are modest, the additional regulatory elements originating from autocrine, endocrine, paracrine, and neuronal signaling are fundamentally important for the final outcome (i.e., insulin secretion) (Berggren et al., 1990).

B. The Inositol Polyphosphates

Figure 1, A and B, shows the structure of inositol and how it can be substituted by phosphates and other groups. The different inositol-related compounds can be divided into several key groups (Fig. 1) composed of two main categories: the water-soluble inositol phosphates and the phospholipids. Further subdivision is then necessary. The lipids are composed of the conventional lipids and the products of phosphatidylinositol 3-kinase. The inositol phosphates consist of two separate groups: the Ca²⁺-regulating second messenger, inositol 1,4,5-trisphosphate [Ins(1,4,5)P₃/IP₃], and the higher inositol polyphosphates. Here, we reviewed these two groups.

With the discovery of Ins(1,4,5)P₃ as the missing link between receptor occupation and Ca²⁺ mobilization in the early 1980s, researchers in the field were ill-prepared for the plethora of metabolites that seemed to arise from this starting compound (Berridge and Irvine, 1984; Irvine and Schell, 2001; Irvine, 2005). In addition to complex deactivation pathways for Ins(1,4,5)P₃, a large group of phosphorylated inositols with up to six phosphate groups occupying the ring were revealed, and more complex pyrophosphates were later found (Fig. 1). An understandable mistake was to search for functions for these novel compounds as second messengers, an undertaking that proved to be fruitless,
with one or two notable exceptions (Shears et al., 2012). However, outside the arena of mammalian cellular biology, higher inositol phosphates already were occupying niches that were far removed from second messenger functions. They were, for example, acting as regulators of avian and reptilian hemoglobin or as a phosphate store in plants and, on this latter basis, as an important element of human nutrition (Irvine and Schell, 2001; Irvine, 2005). Furthermore, some of the most wide-ranging discoveries concerning inositol polyphosphate function have been made in the humble yeast (York, 2006). Despite some insights from Dicytostelium (Stephens and Irvine, 1990; Stephens et al., 1993), it was the study of higher inositol phosphates in yeast that ultimately led to a clear link between the second messenger Ins(1,4,5)P3 and the higher inositol polyphosphates with regard to functional significance (York, 2006). One mammalian cell that is an exception to this rule is the pancreatic β-cell.

It was soon established that the β-cell had a classic Ins(1,4,5)P3-based second messenger system (Best and Malaisse, 1983; Biden et al., 1984, 1987). However, from the late 1990s onward, it became clear that the β-cell had high concentrations of inositol hexakisphosphate (InsP6/IP6), compared with other mammalian cells (60 μM) (Larsson et al., 1997). This observation was echoed later with the discovery in β-cells of a relatively high concentration of the inositol pyrophosphate, diphosphoinositol pentakisphosphate (5′-PPInsP5) (6 μM) (Illies et al., 2007), often termed more loosely as InsP7 or IP7. Of course, relative cellular abundance is not the only deciding factor when establishing functional significance. However, these higher concentrations of inositol polyphosphates were an indication of important things to come.

C. Synergy

A key aspect of this review is the close relationship between new insights into inositol polyphosphate function and our better understanding of pancreatic β-cell stimulus-secretion coupling. A good way of introducing this is to examine the critical processes...
involved in insulin stimulus-secretion coupling and whether inositol polyphosphates have a role. An obvious starting point would be the control of cytoplasmic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)). In the β-cell, this is regulated by both entry through voltage-gated Ca\(^{2+}\) channels and release from intracellular stores. These two mechanisms of controlling [Ca\(^{2+}\)]\(_i\) are regulated by two inositol polyphosphates, the former by InsP\(_6\) (Larsson et al., 1997) and the latter by Ins(1,4,5)P\(_3\) (Biden et al., 1984). In the process of vesicle trafficking and, particularly, endo- and exocytosis, the higher inositol polyphosphates, including both InsP\(_6\) and 5-PP-InsP\(_5\), have important roles (Berggren and Barker, 2008; Barker et al., 2009a). These observations serve to reinforce the existence of intimate relationships between inositol polyphosphates and the understanding of pancreatic β-cell regulation.

The remainder of this review expands on the aforementioned areas of synergy and is divided into three main arenas: 1) Ins(1,4,5)P\(_3\) and its influence on Ca\(^{2+}\) signaling, specifically in a cell in which [Ca\(^{2+}\)]\(_i\) is principally increased by plasma membrane-standing voltage-gated Ca\(^{2+}\) channels; 2) higher inositol polyphosphates, including both InsP\(_6\) and 5-PP-InsP\(_5\), have important roles (Berggren and Barker, 2008; Barker et al., 2009a). These observations serve to reinforce the existence of intimate relationships between inositol polyphosphates and the understanding of pancreatic β-cell regulation.

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II. The Role of Inositol 1,4,5-Trisphosphate in β-Cell Ca\(^{2+}\) Regulation

The regulation of [Ca\(^{2+}\)]\(_i\) is a vast subject (Berridge, 2009), especially in cells as complex as pancreatic β cells. There are several important reviews covering this topic (Hellman et al., 1992; Berggren et al., 1993; Henquin et al., 1998; Niki, 1999; Bergsten, 2002; Tengholm and Gylfe, 2009; Yamazaki et al., 2010; Ramadan et al., 2011). Our focus in this review will be on the particular function of the inositol phosphate Ins(1,4,5)P\(_3\). There has always been a degree of ambiguity about the role of Ins(1,4,5)P\(_3\)-induced Ca\(^{2+}\) release in a cell in which the final executive function is to secrete insulin, a process that is driven by Ca\(^{2+}\) entry via voltage-dependent Ca\(^{2+}\) channels. However, the current view is that the central events of glucose-induced insulin secretion are modulated by additional signals, from neuronal, autocrine, and paracrine sources in the islet, and longer-range regulation by factors, such as incretins (Tengholm and Gylfe, 2009; Yamazaki et al., 2010). These secondary inputs, together with the primary elevation in glucose level, are driven by an important physiologic event, namely the body’s integrated response to a meal. Understanding of this has been further complicated by the appreciation that the structure and, thus, the paracrine/neuronal interactions of the human β-cell are strikingly different from the rodent cells on which so much of our knowledge is based (Cabrera et al., 2006; Jacques-Silva et al., 2010; Rodriguez-Diaz et al., 2011a,b). Therefore, extrapolation of many of the findings regarding inositol polyphosphates in rodent studies need to be carefully filtered if they are to be applied to the study of a human disease (i.e., diabetes).

With regard to Ins(1,4,5)P\(_3\), a central question is what role the β-cell Ins(1,4,5)P\(_3\) plays and, therefore, the Ca\(^{2+}\) stores that it controls. A subsidiary question is whether changes in Ins(1,4,5)P\(_3\) drive intracellular Ca\(^{2+}\) changes, especially oscillations, or whether they are secondary to them. We have covered much of this information in previous reviews (Barker et al., 2002; Barker and Berggren, 2008).
Inositol Polyphosphates in Pancreatic β-Cells

Berggren and Barker, 2008), and we now focus on important insights gained in the intervening time. To resolve these questions, we need to examine the various contexts in which Ins(1,4,5)P3 may play a role: 1) the influence of Ins(1,4,5)P3 under basal conditions; 2) the generation of Ins(1,4,5)P3 after the engagement of G-protein–coupled receptors (GPCRs) as a consequence of phospholipase (PLC) activation; 3) changes in Ins(1,4,5)P3 in response to glucose stimulation and how this synergizes with the input from GPCRs; 4) the relationship between oscillatory changes in Ins(1,4,5)P3 and other oscillatory changes occurring in the β-cells; and 5) the Ins(1,4,5)P3 receptor (IP3R), which is now regarded as the master conductor of Ca2+ in cells, but in which the channel-forming component constitutes only a small fraction of the total protein.

A. The Influence of Ins(1,4,5)P3 Under Basal Conditions

In many cells, without any obvious receptor occupancy, basal levels of Ins(1,4,5)P3 exist, and the same is true for pancreatic β-cells. Although degradation of higher inositol phosphates to Ins(1,4,5)P3 can occur in special organisms (e.g., Dictyostelium) (Van et al., 1995), in the case of most mammalian cells, this Ins(1,4,5)P3 pool is thought to reflect constitutive PLC activity, perhaps combined with a retarded breakdown resulting from the inhibition of the 5-phosphatase by glycolytic intermediates (Rana et al., 1986; Rana et al., 1987). What is the function of this basal Ins(1,4,5)P3?

During the investigation of a poorly glucose-responsive β-cell line, it was noted that an insulin secreting variant of HIT cells have significantly reduced basal [Ca2+], (Yu et al., 2003). Basal Ins(1,4,5)P3 was artificially increased by expressing the normally endoplasmic reticulum (ER)–confined multiple inositol polyphosphate phosphatase (MIPP, Minpp1) (Craxton et al., 1997) in the cytosol (cyt-MIPP), where it could degrade higher inositol polyphosphates, mainly Ins(1,3,4,5,6)P5 to Ins(1,4,5)P3. Expression of cyt-MIPP restored [Ca2+]i to normal basal levels (Yu et al., 2003), suggesting that basal Ins(1,4,5)P3 could be an important element in setting basal [Ca2+]i. However, more striking was the recovery of glucose-induced increases in [Ca2+]i. The renewed functionality appeared to be dependent on the operation of both Ins(1,4,5)P3- and ryanodine-sensitive stores in a process that resembled Ca2+-induced Ca2+ release (CICR) (Fig. 3A). In particular, by controlling basal [Ca2+]i, Ins(1,4,5)P3 was able to influence the threshold required by the ryanodine receptor to function in its capacity to drive Ca2+-induced Ca2+ release. An important message from these experiments is that the resting state of the intracellular Ca2+ handling machinery is an important platform for the development of complex signaling patterns in stimulated cells. However, the key point is that, even under basal conditions, Ins(1,4,5)P3 has a profound influence on [Ca2+]i and expands this role during β-cell stimulation, both via membrane-standing GPCRs and glucose.

No regulatory event in the β-cell should be considered in isolation, and to aid our understanding of this complex system, we must first understand the role of the individual elements and, then, the role of the classic GPCRs in the regulation of intracellular [Ca2+]i, particularly via Ins(1,4,5)P3.

B. Ins(1,4,5)P3 Generated from GPCRs and its Role in β-Cells

The pancreatic β-cell, in common with all mammalian cells, has its intracellular Ca2+ stores regulated by the second messenger Ins(1,4,5)P3. After the occupancy of a GPCR by its ligand, a sequence is initiated in which G-proteins are activated and subsequently drive the breakdown of PtdIns(4,5)P2 by PLC, thus generating Ins(1,4,5)P3 and diacylglycerol (DAG) (Berridge and Irvine, 1984). This canonical pathway, when activated in isolation, has, at best, a modest effect on the promotion of insulin secretion (Berggren and Barker, 2008; Tengholm and Gylys, 2009; Yamazaki et al., 2010). Indeed, some have reported inhibitory effects on secretion when PLC isoforms are overexpressed (Ishihara et al., 1999), although such over-expression would concomitantly lead to a severe depletion of PtdIns(4,5)P2, a necessary cofactor mediating exocytosis (Olsen et al., 2003). Regardless of these caveats, agonists operating through GPCRs have clear physiologic roles in the stimulus-secretion coupling of the β-cell. For example, they implement both neuronal stimulation, via acetylcholine, and autocrine feedback, via ATP (Gilon and Henquin, 2001; Tengholm and Gylys, 2009; Jacques-Silva et al., 2010). However, under physiologic conditions, the GPCR pathways do not operate in isolation, but in combination with other insulin secretagogues, notably glucose. We examine this synergy in the next section of the review. However, to understand how Ins(1,4,5)P3 generation by GPCR acts collaboratively with classic glucose stimulation, it is necessary to examine the role of GPCR-mediated Ins(1,4,5)P3 production in isolation.

A clear indication of the physiologic relevance of PLC-mediated Ins(1,4,5)P3 generation in β-cells comes from two studies. In the first, acetylcholine-activated, PLC-linked M3 receptors were selectively knocked out in mouse pancreatic β-cells (Gautam et al., 2006) and, in the second, depletion of the Ins(1,4,5)P3 receptor after the knockout of ankyrin B (Healy et al., 2010). The phenotype of both these interventions was a marked curtailment of glucose-induced insulin secretion. Although it is important that, downstream of M3 receptor occupancy, lies a myriad of signal transduction pathways (Kong and Tobin, 2011), and not just the production of Ins(1,4,5)P3, the second study emphasizes that Ins(1,4,5)P3 plays an important role. These studies are an important reminder of the
significance of classic-receptor occupancy in a cell in which glucose action has been considered to be preeminent.

Recent studies on the generation of Ins(1,4,5)P₃ in pancreatic β-cells have relied on the indirect measurement of Ins(1,4,5)P₃ by green fluorescent protein (GFP)/enhanced GFP–tagged reporter constructs based on the PtdIns(4,5)P₂-binding PH-domain of PLCδ (Tengholm and Gylfe, 2009; Wuttke et al., 2010).

Under basal conditions, the construct binds to plasma membrane (PM) PtdIns(4,5)P₂. When endogenous PLC is activated and PtdIns(4,5)P₂ breaks down to Ins(1,4,5)P₃, fluorescence is lost from the PM. This loss of fluorescence is measured using either confocal- or total internal reflection fluorescence microscopy. A concern about this approach is its indirectness.

**Fig. 3.** The role of Ins(1,4,5)P₃ in β-cell [Ca²⁺]ᵢ regulation. How Ins(1,4,5)P₃ may be involved in the regulation of [Ca²⁺]ᵢ under several different scenarios. (A) Basal. Under basal conditions, it is likely that the existing concentration of Ins(1,4,5)P₃, derived from the basal activity of PLC, is required to maintain the ER Ca²⁺ store in a condition of readiness for subsequent stimulatory events. For example, a minimum [Ca²⁺]ᵢ is required to maintain the functionality of the ryanodine receptor (RyR).

(B) Stimulation of G-protein receptors by agonists (in this case acetylcholine) institutes activation of PLC and the formation of Ins(1,4,5)P₃. The receptor can also activate, in a G-protein–independent manner, an Na⁺ channel; however, the opening of this channel is insufficient to drive membrane depolarization to the extent that L-type voltage-dependent Ca²⁺ channels are open. The inset depicts the complex interrelationship between G-protein coupled PLC activation (1) and Ca²⁺-driven PLC activation (2 and 3). Already within the first 30 seconds, there is sufficient Ca²⁺ liberated from intracellular stores to further drive PLC activity. (C) Glucose can also stimulate the formation of Ins(1,4,5)P₃, and this is thought to be secondary to Ca²⁺ influx through voltage-dependent Ca²⁺ channels (VDCCs). This means that glucose can stimulate Ins(1,4,5)P₃ generation in the absence of GPCR engagement. However, as with insulin secretion, there is a feedback loop, this time driven by the ATP released concomitantly with insulin exocytosis. This ATP feeds back on a different GPCR again driving Ins(1,4,5)P₃ signaling. In vivo, the signaling through M3 receptors would be either pre-empt or at least be concurrent with glucose action, and thus, the in vivo scenario regarding Ins(1,4,5)P₃ generation is very complex indeed. One important consequence of the simultaneous engagement of M3 receptors and glucose stimulation is that the activation of the Na⁺ current by the M3 receptor is now able to further enhance the depolarization driven by the closure of the K_ATP channels, leading to a stronger Ca²⁺ influx. (Tengholm and Gylfe, 2009; Wuttke et al., 2010).
it is a good assay of PLC activity, it does not directly report on the subsequent changes in Ins(1,4,5)P₃ concentration that are regulated by both PLC generation and subsequent Ins(1,4,5)P₃ metabolism. Unfortunately, direct reliable assays using fluorescence resonance energy transfer generation after Ins(1,4,5)P₃ binding have had poor sensitivity, although a more recent candidate looks to be more promising (Tanimura et al., 2009). Despite the aforementioned caveat about the assay’s indirect measurement of Ins(1,4,5)P₃, the data on Ins(1,4,5)P₃ changes obtained by this technique are quite similar to historical data gained by direct Ins(1,4,5)P₃ measurement (Tengholm and Gylfe, 2009). The clear advantage of this fluorescent reporter approach is its ability to monitor Ins(1,4,5)P₃-related events in single cells simultaneously with other important parameters, especially the monitoring of [Ca²⁺]i. Studies using this approach have revealed new levels of complexity, even at the level of the simple and classic GPCR-mediated liberation of Ca²⁺ from Ins(1,4,5)P₃-sensitive stores. Even greater complexity arises when we consider glucose stimulation, which we discuss in the next section.

The original studies on the engagement of β-cell GPCRs with maximal agonist concentrations, in line with other cell systems, demonstrate parallel, biphasic Ins(1,4,5)P₃ and [Ca²⁺]i increases. The initial phase is marked by both a rapid increase in Ins(1,4,5)P₃ and [Ca²⁺]i, and a lower sustained phase of both molecules (Fig. 3B).

Some caution needs to be applied when considering data based on maximal agonist concentration, because Cobold and Cuthbertson only discovered agonist-dependent changes in the frequency of [Ca²⁺]i oscillations after GPCR activation of PLC, when using physiologic agonist concentrations (Cobold and Cuthbertson, 1990). Physiologic concentrations are often dramatically lower than the maximum effective concentrations extrapolated from the dose-response curve. Unfortunately, there is less information in β-cells about the oscillatory behavior of [Ca²⁺]i when stimulated with lower, physiologic concentrations of agonists. However, because the majority of published studies use high agonist concentrations, most information on the PLC system in β-cells comes from them.

Studies using the aforementioned GFP-PLCδ-PH domain reporter technique, in conjunction with classic Ca²⁺-sensitive fluorescent dyes, also indicate two phases of PLC activity, consistent with the older published data using direct Ins(1,4,5)P₃ measurements (Tengholm and Gylfe, 2009). There is an initial rapid increase in Ins(1,4,5)P₃ peaking at ~30 seconds, followed by a second lower sustained phase Fig. 3B. In both phases, there is some dependency on the Ca²⁺ released from intracellular stores to drive PLC. However, with use of either 2-APB or La³⁺, blockers of store-mediated Ca²⁺ entry or nonspecific Ca²⁺ entry, the sustained but not the initial phase of PLC activity is blocked (Thore et al., 2005). There is therefore a feed-forward activation of PLC, through both release of Ca²⁺ from intracellular stores and capacitative Ca²⁺ entry. In the β-cell, entry through store-operated channels is modest (Tengholm and Gylfe, 2009), and it has been proposed that the efficacy of this Ca²⁺ is enhanced by a close proximity between Ca²⁺ entry channels and PLC (Tengholm and Gylfe, 2009). The modest increase in [Ca²⁺], from store entry may reflect a reduced level of STIM1 in β-cells. STIM1, together with Orai channels, mediate the store-operated Ca²⁺ entry in cells (Srikanth and Gwack, 2012), including β-cells (Tamarina et al., 2008; Tengholm and Gylfe, 2009). There is no direct proof of reduced STIM1 expression in β-cells, but recent data from other cell types suggest a reciprocal relationship between store-mediated Ca²⁺ entry and entry through Ca₁.2 voltage-dependent Ca²⁺ channels. STIM1 blocks the voltage-dependent Ca²⁺ channels, making them silent (Park et al., 2010; Wang et al., 2010). The fact that the β-cell has active and functionally critical voltage-dependent channels suggests that STIM1 concentration is lower in these cells. In other cell systems, physical coupling between TRP channels and PLC has been shown, but how this is arranged in the β-cell is currently poorly characterized.

In terms of the PLC isoform(s) that may be activated, classically, PLCβ is coupled to GPCRs, whereas PLCδ, because of its higher Ca²⁺ sensitivity, is proposed to drive Ca²⁺-stimulated PLC activation. However, at least in some insulin-secreting cell lines, there is also evidence for Ca²⁺-driven activation of the PLCβ isoform (Kelley et al., 2001). These data should be considered carefully, however, because the conclusions about isoform involvement are based on the transfection of exogenous PLCs. Nonetheless, PLC β, δ, and γ have been detected in β-cells (Tengholm and Gylfe, 2009; Yamazaki et al., 2010). Although PLCγ is not activated directly by Ca²⁺, it seems to play a role in [Ca²⁺]i regulation (Patterson et al., 2005).

An interesting perspective on acetylcholine/carbachol regulated [Ca²⁺], and Ins(1,4,5)P₃ has come from the characterization of the islets from mice in which the β₃ subunit of the L-type voltage-dependent Ca²⁺ channel has been globally knocked out (Berggren et al., 2004). There are many interesting facets to this phenotype, because the overall result is apparently an enhanced sensitivity to glucose stimulation and subsequent release of insulin. Other aspects are discussed later, but one key observation is relevant to our current discussion. In comparing the islets from these knockout animals with those of controls, carbachol-stimulation led to both an enhanced initial increase in [Ca²⁺], and an enhanced increase in Ins(1,4,5)P₃ within the same period (30 seconds stimulation). Removal of extracellular Ca²⁺ normalized the response to that of the islets from the control animals; thus,
some form of Ca\(^{2+}\) entry is presumed to be necessary (unpublished data). Furthermore, the Ca\(^{2+}\) entry could be blocked with a specific L-type Ca\(^{2+}\) channel inhibitor, nimodipine (unpublished data). At first sight, the participation of the L-type Ca\(^{2+}\) channel seems to be important, but under standard conditions, no changes in Ca\(^{2+}\) channel parameters were observed in the knockout animal. Historically, engagement of muscarinic receptors in β-cells has been shown to slightly depolarize cells after activation of an Na\(^+\) current (Henquin et al., 1988). However, under the basal glucose concentration that was used in the β\(_3\) knockout study, the depolarization would not be sufficient to open voltage-gated channels (Henquin et al., 1988). What other possibilities exist to explain these data? One suggestion is that nimodipine is blocking another Ca\(^{2+}\) entry pathway. Indeed, dihydropyridines, such as nimodipine, can block store- or receptor-operated Ca\(^{2+}\) channels (Harper et al., 2003), but this usually occurs at higher doses of the inhibitor than was used in the knockout study. Furthermore, as we have stated earlier, store-operated Ca\(^{2+}\) entry occurs after the initial increase in Ca\(^{2+}\) and Ins(1,4,5)P\(_3\) and, thus, is unlikely to contribute to the increase in [Ca\(^{2+}\)]\(_i\) occurring within the first 30 seconds. Several other possible explanations can be advanced to explain how β\(_3\) knockout is blocking the carbachol response. For example, the lifetime of the G\(_{q}\)α subunit, which drives PLC activity, could be sustained by the increased binding of the inactivating Gβγ to either the alpha subunit of the L-type channel (Yang and Berggren, 2005) or some other intracellular target (Berggren et al., 2003). The increased binding to the channel or another unidentified protein is made possible because of the vacation of the binding site by β\(_3\). In any event, the β\(_3\) knockout model is intriguing, because it starts to expose little understood aspects of classic GPCR activation of PLC and the mobilization of intracellular Ca\(^{2+}\), indicating that there is still much to learn about even this basic system.

C. Changes in Ins(1,4,5)P\(_3\) in Response to Glucose Stimulation

When we consider Ins(1,4,5)P\(_3\) concentration increases driven by glucose stimulation, a new level of complexity emerges as both GPCR- and Ca\(^{2+}\)-driven PLC activation occur, without addition of exogenous GPCR ligands. We describe how this occurs below.

From the earliest work in this field, it was clear that glucose alone could drive the increase in Ins(1,4,5)P\(_3\) formation (Best and Malaisse, 1983; Biden et al., 1987), although this was often a modest response, compared with, for example, carbachol-driven changes. Although there were a number of suggestions of how glucose might increase intracellular Ins(1,4,5)P\(_3\), the major explanation to emerge was the idea that the Ins(1,4,5)P\(_3\) was derived from Ca\(^{2+}\) activation of PLC, secondary to Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels (Barker et al., 2002). However, the original experiments suggested that this modest increase in Ins(1,4,5)P\(_3\) was not wholly dependent on the presence of extracellular Ca\(^{2+}\) (Biden et al., 1987). This glucose-dependent, Ca\(^{2+}\)-independent generation of Ins(1,4,5)P\(_3\) may be derived from either the arrested breakdown of Ins(1,4,5)P\(_3\) by glycolytic metabolites (Rana et al., 1986; Rana et al., 1987) or depolarization-induced activation of PLC (Henquin et al., 1998; Bergsten, 2002; Thore et al., 2004; Tamarina et al., 2005; Tengholm and Gylfe, 2009). Because glucose stimulation alone can drive PLC activity, the same series of events shown Fig. 3B with the Ins(1,4,5)P\(_3\) and the Ca\(^{2+}\) stores can occur with glucose stimulation alone (Fig. 3C).

Glucose stimulation is more complex than the engagement of the M3 muscarinic receptor (see section IB; Fig. 3B), because the influx of Ca\(^{2+}\) through voltage-dependent Ca\(^{2+}\) channels also leads to the release of both insulin and ATP from insulin-containing granules. This ATP, in turn, can act through purinergic GPCRs to also stimulate PLC and generate Ins(1,4,5)P\(_3\), again leading to the complex interplay of stores and capacitive Ca\(^{2+}\) entry (Tengholm and Gylfe, 2009). These events induced by ATP are temporally separated from the events stimulated by glucose. Therefore, the Ins(1,4,5)P\(_3\) generated and the Ca\(^{2+}\) subsequently mobilized may influence signaling events distinct in time from those generated by activation of M3 GPCRs and/or glucose-induced Ca\(^{2+}\) influx. The link between released ATP and PLC activation is mediated through P2Y receptors in rodents (Petit et al., 2009). An added complication is that, in contrast to rodents, human β cells have ionotropic P2×3 receptors (Jacques-Silva et al., 2010) and not P2y receptors. P2×3 receptors are not linked to PLC activation but are membrane ion channels that, on engagement, mediate the influx of extracellular Ca\(^{2+}\) (Jacques-Silva et al., 2010). Ca\(^{2+}\) entry through plasma membrane standing-channels could influence a local environment that is different from the one that Ca\(^{2+}\)-mobilized from intracellular Ca\(^{2+}\) stores reaches. Therefore, it is possible that there is some diversity in downstream signaling between rodent and human islets. Indeed purinergic stimulation in rodents has been described to both stimulate and inhibit insulin secretion (Petit et al., 2009), whereas in humans, it seems to solely enhance insulin secretion (Jacques-Silva et al., 2010).

The final issue to consider in relation to glucose-stimulated increases in Ins(1,4,5)P\(_3\) is how these glucose-induced changes act in concert with the Ins(1,4,5)P\(_3\) produced by the acetylcholine-mediated activation of M3 GPCRs (Gilom and Henquin, 2001). This scenario is more representative of the physiologic situation when the β-cell responds to multiple inputs after the consumption of food. When acetylcholine or its analog, carbachol, are applied simultaneously with glucose stimulation, there is a synergistic effect on insulin...
secretion (Biden et al., 1987). One reason for this is the muscarinic-driven opening of the Na\(^+\) channel, which partially depolarizes the membrane (Henquin et al., 1988). As stated in section I.B, under basal glucose conditions, this depolarization is insufficient to drive Ca\(^{2+}\) entry through voltage-dependent Ca\(^{2+}\) channels. However, this modest depolarization becomes significant in combination with the glucose-induced depolarization, resulting in the closure of K\(_{ATP}\) channels. The combined depolarization is greater than that elicited by blockade of the K\(_{ATP}\) channels alone, resulting in an enhanced Ca\(^{2+}\) entry through voltage-dependent Ca\(^{2+}\) channels. Thus, a synergistic effect is created between GPCR activation and glucose metabolism. It has been suggested that part of the Na\(^+\) current may be attributable to Na\(^+\) entry through the M3 receptor (Swayne et al., 2010). An independent Na\(^+\) tetrodotoxin-insensitive channel has now been identified, sodium leak channel, nonselective (NALCN), which at least in part, is likely to be responsible for this phenomenon (Rolland et al., 2002b; Swayne et al., 2010) and is directly coupled to M3 receptors (Swayne et al., 2010).

We have already described above the data from the \(\beta\)-cell–specific M3 receptor knockout mouse, which underscores the importance of the synergy between parasympathetic input and glucose increases in driving insulin secretion (Gautam et al., 2006). A more recent report reinforces this idea from a different perspective. In both cases, an impairment of glucose-stimulated insulin secretion results in a type 2 diabetic (T2D) phenotype. The latest report comes from work on the ankyrin B knockout mouse (Healy et al., 2010). Ankyrin B is only expressed in the pancreatic \(\beta\)-cells and not in other islet cells, which aids interpretation of the results. This knockout mouse has a similar phenotype to the \(\beta\)-cell–specific M3 receptor knockout mouse with regard to its dampened cholinergic augmentation of glucose-stimulated insulin release. In this case, the IP3R specifically is involved. Because homozygous ankyrin B knockout mice do not survive to adulthood, experiments were performed using heterozygous animals with half the copy number of ankyrin B. In these animals, there was a severely blunted and delayed increase in \([Ca^{2+}]_i\) in response to carbachol, both in the presence and absence of extracellular Ca\(^{2+}\). There was a similarly blunted response to the carbachol-enhanced, glucose-stimulated secretion of insulin in dynamic insulin release assays. Strikingly, even glucose-stimulated insulin secretion alone was compromised, highlighting the importance in glucose-induced Ins(1,4,5)P\(_3\) signaling in exocytosis. A key defect in these cells was a halved amount of the IP3R. This suggests that, when ankyrin-B is absent, it is no longer able to target the IP3R correctly, leading to its destruction. Furthermore, a loss of function mutation of ankyrin B (R1788W) was found in a significant number of patients with T2D. This study is helpful in our thinking about the role of Ins(1,4,5)P\(_3\), because the knockout of the M3 receptor affects several different signal transduction pathways besides the generation of Ins(1,4,5)P\(_3\). However, the ankyrin B study suggests that the downstream production of Ins(1,4,5)P\(_3\) from the M3 receptor and its modulation of intracellular Ca\(^{2+}\) stores is likely to be a key element of the M3 knockout phenotype.

Another aspect of Ins(1,4,5)P\(_3\) involvement in the regulation of Ca\(^{2+}\) comes with the observation that Ins(1,4,5)P\(_3\) can indirectly activate a Ca\(^{2+}\)-activated, inwardly rectifying K\(^+\) channels (Ammala et al., 1991). This becomes more significant when we discuss the influence of Ins(1,4,5)P\(_3\) on Ca\(^{2+}\) oscillations in the next section. This topic of oscillatory phenomena in the regulation of \(\beta\)-cells represents the final level of complexity involving Ins(1,4,5)P\(_3\).

D. A Role for Ins(1,4,5)P\(_3\) in \([Ca^{2+}]_i\) Oscillations

Oscillations in cellular messengers, including Ca\(^{2+}\), are an established phenomenon in cell biology. It has particular importance in the \(\beta\)-cell, because besides \([Ca^{2+}]_i\), electrical activity, metabolism, cAMP, and insulin, are subject to dynamic change in a synchronized manner (Tengholm and Gylfe, 2009). We consider specifically the relationship of Ins(1,4,5)P\(_3\) to \([Ca^{2+}]_i\) oscillations in this section.

A notable breakthrough in mammalian Ca\(^{2+}\) signaling was the observation made in hepatocytes that, at lower physiologic concentrations of agonists, the oscillation frequency (but not the amplitude) of Ca\(^{2+}\) spikes was directly proportional to agonist concentration (Cobbold and Cuthbertson, 1990). An important question, of course, is whether oscillations in \([Ca^{2+}]_i\) are driven by parallel oscillations in Ins(1,4,5)P\(_3\). If this is the case, it would imply an oscillatory behavior of PLC or an oscillatory behavior of Ins(1,4,5)P\(_3\) metabolism. In the literature, there are examples of \([Ca^{2+}]_i\) oscillations with or without parallel oscillations in Ins(1,4,5)P\(_3\) (Berridge, 2007). This seems to be dependent on the system examined (Berridge, 2007), although, as we have noted above, some of the evidence has come from the indirect measurement of PLC activity with use of the GFP-PLCdelta PH domain (Tengholm and Gylfe, 2009). In summary, it is now generally understood that Ins(1,4,5)P\(_3\) does not need to oscillate to generate oscillations in \([Ca^{2+}]_i\), even when we consider the simplest system without the involvement of voltage-dependent Ca\(^{2+}\) entry. The oscillatory behavior can arise from the complex interplay between store-filling, the IP3R sensitivity to luminal Ca\(^{2+}\), and the process of Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR). Berridge has called this the “store- or luminal-loading model of \([Ca^{2+}]_i\) oscillations” (Berridge, 2007, 2009). We consider...
this model later, because we believe that it has some bearing in trying to understand what is happening in pancreatic β-cells.

Narrowing our focus back to pancreatic β-cells, an early observation was that oscillations in \( \text{Ins}(1,4,5)\text{P}_3 \) could accompany oscillations in \([\text{Ca}^{2+}]_i\) (Barker et al., 1994). The β-cell is considered to have two types of oscillation in \([\text{Ca}^{2+}]_i\): fast oscillations, which are strongly linked to plasma membrane electrical activity, and slow oscillations, which correlate better with oscillations in metabolism and, more importantly, oscillations in insulin release (Hellman et al., 1992; Henquin et al., 1998; Bergsten, 2002; Rolland et al., 2002a; Thore et al., 2004; Tamarina et al., 2005). In these early experiments, a population of isolated pancreatic β-cells was artificially synchronized, after which several parallel slow oscillations in \([\text{Ca}^{2+}]_i\) and \(\text{Ins}(1,4,5)\text{P}_3\) were observed (Barker et al., 1994). Unfortunately, the system did not have enough temporal resolution to clearly delineate whether \(\text{Ins}(1,4,5)\text{P}_3\) oscillations drove \([\text{Ca}^{2+}]_i\) oscillations or vice versa. More recently, the use of GFP-PLCδ1 PH domain reporters has led to the observation of similar parallel oscillations in \(\text{Ins}(1,4,5)\text{P}_3\) and \([\text{Ca}^{2+}]_i\) under both slow and faster oscillatory patterns (Henquin et al., 1998; Bergsten, 2002; Thore et al., 2004; Tamarina et al., 2005; Beauvois et al., 2006; Tengholm and Gylfe, 2009). In some of these studies, the temporal resolution has been sufficiently good to demonstrate that \([\text{Ca}^{2+}]_i\) oscillations drive \(\text{Ins}(1,4,5)\text{P}_3\) oscillations and not the other way round. Does this mean that in the β-cell \(\text{Ins}(1,4,5)\text{P}_3\) and, thus, that the mobilization of \([\text{Ca}^{2+}]_i\) from intracellular stores is completely subservient to \([\text{Ca}^{2+}]_i\) entry? A consideration of the two previous sections of this article would indicate that the situation is unlikely to be so clear-cut because of the intricate feedback loops existing in even the simplest scenarios involving \(\text{Ins}(1,4,5)\text{P}_3\), \([\text{Ca}^{2+}]_i\) stores and \([\text{Ca}^{2+}]_i\) entry.

Figure 4 shows the Berridge model of store- or luminal-loading model of calcium oscillations (Berridge, 2007, 2009), which we will now try and integrate into our understanding of β-cell \([\text{Ca}^{2+}]_i\) oscillations. This model is dependent on the following experimentally verified elements: the IP3R luminal \([\text{Ca}^{2+}]_i\) sensor, store-operated \([\text{Ca}^{2+}]_i\) entry, the mobilization of \([\text{Ca}^{2+}]_i\) from intracellular stores and the process of CICR. The way that Berridge has envisioned this is as follows. Under basal conditions and at low agonist doses, there is not sufficient \(\text{Ins}(1,4,5)\text{P}_3\) to mobilize \([\text{Ca}^{2+}]_i\), but there is enough agonist to promote \([\text{Ca}^{2+}]_i\) entry and a filling of the stores. Increasing \([\text{Ca}^{2+}]_i\) in the stores leads to spontaneous \([\text{Ca}^{2+}]_i\) release (spikes), because the increased \([\text{Ca}^{2+}]_i\) changes the conformation of the IP3R through its luminal \([\text{Ca}^{2+}]_i\) sensor. This structural change makes the IP3R more sensitive to \(\text{Ins}(1,4,5)\text{P}_3\), leading to a release of \([\text{Ca}^{2+}]_i\). There is then an amplification of the increasing phase of the \([\text{Ca}^{2+}]_i\) spike driven by CICR.

What then is the explanation that Berridge offers for the change in frequency of the oscillations seen after higher agonist concentrations? He suggests that the increased agonist concentrations lead to a higher rate of store filling, because only at a high \([\text{Ca}^{2+}]_{\text{ER}}\) will \([\text{Ca}^{2+}]_i\) spiking occur. There is a significant amount of literature that supports the idea that the intracellular stores are important for at least sustaining \([\text{Ca}^{2+}]_i\) oscillations, but this proposal takes this idea one step further by attempting to also explain changes in oscillatory frequency. The key question for our current review is whether this has a role to play in understanding of the regulation of \([\text{Ca}^{2+}]_i\) oscillations in pancreatic β-cells and islets.

A good place to start, perhaps, is an examination of those islets that oscillate, compared with those that do not, in an otherwise identical preparation. Such a scenario was investigated relatively recently (Jahanshahi et al., 2009). First, islets that did not oscillate had high basal \([\text{Ca}^{2+}]_{\text{ER}}\), and reduced \([\text{Ca}^{2+}]_i\) responses to glucose stimulation. However, the crux of the difference appeared to be a decreased content of ER \([\text{Ca}^{2+}]_{\text{ER}}\), rather than in any defects in mitochondrial membrane potential, plasma membrane channels (including L-type \([\text{Ca}^{2+}]_i\) channels and KATP channels), or cell death rates. Of interest, in other systems, it has been reported that \(\text{Ins}(1,4,5)\text{P}_3\)-regulated ER stores are important for the sustaining of oscillations (Yano et al., 2004). These data seem to fit well with the aforementioned Berridge hypothesis.

Fig. 4. The luminal \([\text{Ca}^{2+}]_i\) model of \(\text{Ins}(1,4,5)\text{P}_3\)-induced oscillations in \([\text{Ca}^{2+}]_i\). The figure shows the luminal \([\text{Ca}^{2+}]_i\) model of generating oscillations in \([\text{Ca}^{2+}]_i\), proposed by Berridge (2007). For a detailed discussion about how this may impact on β-cell \([\text{Ca}^{2+}]_i\) oscillations, see Section III.D. In brief, the model proposes that, after GPCR engagement with low, but physiologic concentrations of agonist, \(\text{Ins}(1,4,5)\text{P}_3\) is released, but does not achieve sufficient concentrations to activate the IP3R. Therefore, there is lag phase (L). During this time, there is a gradual build up in luminal \([\text{Ca}^{2+}]_i\), through increased uptake into the stores, from extracellular \([\text{Ca}^{2+}]_i\) entry. When a threshold \([\text{Ca}^{2+}]_{\text{ER}}\) is reached, the sensitivity of the IP3R to \(\text{Ins}(1,4,5)\text{P}_3\) is enhanced, leading to activation of the receptor and the release of \([\text{Ca}^{2+}]_i\) from the stores in the form of a spike. The luminal concentration consequently decreases, and the cycle is repeated as the stores refill. The change in frequency from slow to fast is then dependent on the time that it takes to refill the stores. More efficient store filling will lead to faster oscillations.
Another scenario that we have already introduced is the β3 knockout mouse with its enhanced frequency of [Ca2+]i oscillations. In this model, an increased Ca2+ mobilization is observed after stimulation with the GPCR agonist carbachol. Although this enhanced mobilization is dependent on extracellular Ca2+, suggesting that Ca2+ entry plays a role, whatever the source, the greater increase in [Ca2+]i could easily lead to a more rapid filling of stores and, thus, a faster onset of the next oscillation. Examination of the Ca2+ stores of these mice revealed that the rate of loss of Ca2+ from the stores was significantly slower than that of islets from control animals (unpublished data). This could reflect less efficient mobilization (which is unlikely, given the general phenotype) or a more efficient refilling of the pools, shortening the time to the next oscillation. Of interest, when inhibitors of the IP3R are applied to the β3 knockout islets, the [Ca2+]i oscillation frequency of the β3 knockout islets decreases (Berggren et al., 2004).

In summary, Ins(1,4,5)P3 is likely to be important for the sustaining of [Ca2+]i oscillations in pancreatic β-cells, and although it is most likely that Ins(1,4,5)P3 oscillations follow [Ca2+]i oscillations rather than vice versa, the Berridge model suggests that Ins(1,4,5)P3 could play a role. The major caveat with this theory is that there is much evidence to suggest that it is the electrical excitability of the plasma membrane and voltage-driven events that underscore β-cell [Ca2+]i oscillations (Tengholm and Gyfle, 2009). The current common consensus is that the time-keeping and initiation of oscillations is driven by these plasma membrane events and that Ins(1,4,5)P3-dependent Ca2+ events enhance the fundamental oscillations already established by the classic oscillations in electrical activity. Therefore, any information that could link Ins(1,4,5)P3 to membrane electrical activity would save this inositol phosphate from just being a follower of oscillatory events in excitable cells. Of interest, a study from more than 20 years ago addresses this issue specifically.

The end of the [Ca2+]i oscillation is marked by a repolarization of the plasma membrane and an effective silencing of voltage-dependent Ca2+ entry. This repolarization is believed to be performed by inwardly rectifying Ca2+-activated K+ channels. A family of such channels exists in the PM of pancreatic β-cells. Twenty years ago, one channel that was insensitive to the triethylamine inhibition, typical of this family of such channels, was found to be specifically sensitive to the Ins(1,4,5)P3-promoted release of Ca2+ from intracellular stores (Ammala et al., 1991). Thus, Ins(1,4,5)P3 could indirectly influence the electrically driven oscillations in [Ca2+]i. Certainly, evidence from the study of other inwardly rectifying K+ channels suggests that their inhibition can change the frequency of [Ca2+]i oscillations in pancreatic β-cells. These studies leave open the debate about the centrality of Ins(1,4,5)P3 in the regulation of β-cell [Ca2+]i oscillations and reveal an important area for future investigations.

E. The β-Cell Ins(1,4,5)P3 Receptor

The IP3R was already featured several times in our discussions. In fact, comparatively few studies have been performed on the β-cell IP3Rs; therefore, most of our information is from extrapolation from work done on IP3Rs in other systems. We do not discuss these here but highlight what is known specifically about the β-cell IP3R receptors and suggest that the reader refers to several excellent reviews covering this subject (Patterson et al., 2004; Mikoshiba, 2007; Taylor and Prole, 2012).

Evidence for type 1–3 IP3Rs exists in pancreatic β cells. Originally, there was a disproportionate focus on the type 3 receptor, because it was believed to be specifically enriched in β-cells (Hagar and Ehrlich, 2000). However, this perception arose from the use of the rat RIN m5F β-cell line, which is enriched in this receptor and was reinforced by subsequent studies on primary rat β-cells; these had the same dominance as the type 3 receptor (Lee et al., 1999). However, in mouse pancreatic β-cells, the type 1 is as abundant as type 3 (Lee and Laychock, 2001). The actual proportions of these different isoforms may be important in trying to understand β-cell [Ca2+]i oscillations, because in other cells, deletion of one or the other IP3R subtype has resulted in different effects on [Ca2+]i oscillations (Zhang et al., 2011).

Another controversial aspect of β-cell IP3Rs is the notion that IP3Rs are in insulin-containing vesicles and regulate vesicle Ca2+. This area is contentious, because the original study showing this (Blondel et al., 1994) was subsequently questioned. The debate centered around the reliability of the antibody used to localize the receptor (Ravazzola et al., 1996). In other cell systems, there now seems to be a clear indication of IP3R in secretory granules (Yoo and Hur, 2012), which at least leaves this idea open.

Most of the focus related to the IP3R in the β-cell, in common with many other cell types, has been directed at the regulation of [Ca2+]i. However, it is sobering to realize that the Ca2+ pore-forming domain represents only ~5% of the total sequence of the protein (Patterson et al., 2004; Mikoshiba, 2007). Although there is a portion of the molecule that is exposed to the ER lumen, the vast majority of the protein forms a massive porous superstructure, which in addition to possessing many sites for the functional regulation of the channel, has a large number of other proteins associated with it. One yeast 2 hybrid screen trapped more than 300 proteins (Patterson et al., 2004), and although such approaches can give false positives, to date, more than 25 proteins have been rigorously
demonstrated to be associated with the channel. This includes structural proteins, regulatory proteins, and proteins that seem to use the IP3R as a docking station from which they are liberated to implement diverse cellular functions. When considering this aspect of the IP3R, it is clear that it is a unique master conductor of cellular regulation and may exert many thus far uncharacterized events around cellular \([\text{Ca}^{2+}]_i\) regulation. With few exceptions, there has been little characterization of partner proteins in \(\beta\)-cells. However, the data from both the ankyrin B (Healy et al., 2010) and \(\beta_3\)-knockout mice (Berggren et al., 2004), suggest that there is much to explore in this subject area.

An important consideration when thinking about the pancreatic \(\beta\)-cell is the quest to identify the components in the stimulus-secretion coupling that may contribute to or be affected by the T2D phenotype. We have already described the role that the IP3R may have in human diabetes, which was revealed by studies using the ankyrin B–deficient mouse. Are there other examples of links between IP3Rs and diabetes? There are two examples to which we can refer, both relatively recent. The first links the gene for the type 3 IP3R (ITPR3) to type 1 diabetes (T1D) in the Swedish population (Swedes have one of the highest incidences of T1D worldwide). The original publication suggested an independence of the association from any MHC class II gene (Roach et al., 2006) and, thus, a clear likelihood of the involvement of the IP3R gene. Unfortunately, another group in a subsequent study (Qu et al., 2008) questioned these findings and, in their study, found that the ITPR3 gene polymorphism was attributable to a linkage disequilibrium with HLA class II. A second recent example relates to T2D. After investigation of a transgenic mouse line that exhibited a progressive glucose intolerance, it was shown that the transgenic insertion disrupted 10 loci in chromosome 6, one of which was IP3R1 (Ye et al., 2011). Under a regular diet, the heterozygous transgenic mice developed glucose intolerance, but not insulin resistance, and a decrease in second-phase, glucose-stimulated blood insulin levels, consistent with a reduced insulin release in islets. In this context, of importance, \(\text{Ca}^{2+}\) mobilization from Ins(1,4,5)P\(_3\), a downstream product of Ins(1,4,5)P3, it is nonetheless regulated distinct from Ins(1,4,5)P\(_3\), it is nonetheless regulated in mammalian cells by inositol 1,3,4-trisphosphate [Ins(1,3,4)P\(_3\)], a downstream product of Ins(1,4,5)P3 metabolism. It does this in a unique and elegant fashion (Shears et al., 2012). We first focus on the unique mechanism of regulation of Ins(3,4,5,6)P4 and, then, return to the discussion of its second messenger activity.

Ins(3,4,5,6)P4 is generated by an idiosyncratic enzyme, inositol tetraphosphate kinase, which was originally identified as an Ins(1,3,4)P3 5/6 kinase (Abdullah et al., 1992) and, then, an Ins(3,4,5,6)P4 1-kinase (Tan et al., 1997). In fact, it performs a multitude of functions, including the formation of Ins(1,3,4,5,6)P\(_5\) from Ins(1,3,4)P\(_3\) through either Ins(1,3,4,6)P\(_4\) or Ins(1,3,4,5)P\(_4\) or the formation of Ins(1,3,4,5,6)P\(_5\) from Ins(3,4,5,6)P\(_4\) and vice versa, thus acting as both a kinase and a phosphatase (Fig. 1) (Ho et al., 2002). However, it performs these functions in an atypical manner; thus, for example, the phosphatase activity is not inhibited by ATP as a normal reverse of a kinase reaction would be. The complete mechanism was only recently elucidated after detailed structural information from a crystallographic study of the human enzyme, which has important differences from the structures of the equivalent plant or amoeba enzymes that have been determined previously (Chamberlain et al., 2007). Thus, only the human and, presumably, other mammalian enzymes can use Ins(1,3,4)P\(_3\) to regulate the concentration of Ins(3,4,5,6)P\(_4\) (Chamberlain et al., 2007). The Ins(1,3,4)P\(_3\) is derived sequentially from the metabolism of the second messenger Ins(1,4,5)P\(_3\) through Ins(1,3,4,5)P\(_4\), and thus, its concentration is directly coupled to receptor occupancy. The elevation in Ins(1,3,4)P\(_3\) then drives increases in the metabolically unrelated Ins(3,4,5,6)P\(_4\) (Yang and Shears, 2000). The mechanism of this regulation is believed to be a specialized intersubstrate phosphate transfer, mediated

### III. Higher Inositol Polyphosphates

#### A. Inositol 3,4,5,6-Tetrakisphosphate as a Novel Second Messenger

When we look beyond Ins(1,4,5)P\(_3\) for a second messenger in the realm of the higher inositol polyphosphates, there is perhaps only one serious candidate: inositol 3,4,5,6-tetrakisphosphate [Ins(3,4,5,6)P\(_4\)] (Shears et al., 2012). Other higher inositol polyphosphates have been claimed in this role (Shears et al., 2012); however, because of their existing high concentrations in cells, it is more likely that they function as cellular regulators rather than messengers. Of interest, although Ins(3,4,5,6)P\(_4\) is metabolically distinct from Ins(1,4,5)P\(_3\), it is nonetheless regulated in mammalian cells by inositol 1,3,4-trisphosphate [Ins(1,3,4)P\(_3\)], a downstream product of Ins(1,4,5)P3 metabolism. It does this in a unique and elegant fashion (Shears et al., 2012). We first focus on the unique mechanism of regulation of Ins(3,4,5,6)P4 and, then, return to the discussion of its second messenger activity.
Inositol Polyphosphates in Pancreatic β-Cells

653

by a nucleotide deeply imbedded in the active site of the enzyme. In short, the 1-phosphate of \( \text{Ins}(1,3,4,5,6)P_5 \) is donated to the ADP tightly bound to the enzyme, forming \( \text{Ins}(3,4,5,6)P_4 \) and ATP, the former being released. In a linked subsequent step, this bound ATP donates a phosphate to \( \text{Ins}(1,3,4)P_3 \), and the \( \text{Ins}(1,3,4,6)P_4 \) or \( \text{Ins}(1,3,4,5)P_4 \) that is formed is released. Thus, \( \text{Ins}(1,3,4)P_3 \) acts as an acceptor that enables the cycling of the enzyme back into a form that can again degrade \( \text{Ins}(1,3,4,5,6)P_5 \) to \( \text{Ins}(3,4,5,6)P_4 \). The increase in \( \text{Ins}(3,4,5,6)P_4 \) after receptor occupancy is seen in all mammalian cells studied, including insulin-secreting cells (Shears et al., 2004). In β-cells, stimulation with glucose without receptor occupancy can generate \( \text{Ins}(1,3,4,5)P_3 \) (see section II.C and Fig. 3C). Therefore, it is possible that, in β-cells, \( \text{Ins}(3,4,5,6)P_4 \) can be generated without the engagement of GPCRs. This brings us to the second messenger function of \( \text{Ins}(3,4,5,6)P_4 \).

Shears et al. (2004, 2010) established that \( \text{Ins}(3,4,5,6)P_4 \), within its defined physiologic range and with great specificity, inhibited the CaMKII-regulated chloride channels found largely in the PM of cells. In mammalian cells, this Cl-channel has been identified as the CIC-3 subtype. These channels regulate salt and fluid secretion in epithelial cells and cell volume or electrical excitability in muscle and neuronal cells (Shears et al., 2004, 2012). Unfortunately, the precise mechanism by which \( \text{Ins}(3,4,5,6)P_4 \) inhibits these channels is still unknown, but it is unlikely to be a direct interaction with the channel. In β-cells and, perhaps, in other cell systems, \( \text{Ins}(3,4,5,6)P_4 \) can regulate the homologous CaMKII-activated chloride channels found in the membranes of secretory vesicles (Renstrom et al., 2002). The role of these channels is to provide a counter ion and, thus, charge neutralization during the acidification of the vesicle by the electrogenic H\(^+\)-ATPase. This acidification is considered to be an important step to produce fusion-competent vesicles in the final stages of exocytosis (Hou et al., 2009). This means that \( \text{Ins}(3,4,5,6)P_4 \) acts to inhibit acidification and, thus, exocytosis (Renstrom et al., 2002). Two studies showing the importance of the CIC-3 channel for insulin exocytosis were published in Cell Metabolism (Deriy et al., 2009; Li et al., 2009). These two studies reported that the complete knockout of the CIC-3 gene led to impaired glucose and K\(^+\)-stimulated insulin secretion. However, these studies were questioned on several grounds, including conflicting information between them and a dispute about the presence of CIC-3 in insulin-secretory vesicles, rather than other vesicle types (Jentsch et al., 2010). Both the original groups were, however, able to provide a robust defense, including additional data supporting their positions in the same issue of the journal. These studies highlight that the CIC-3 is involved in several distinct processes leading to secretion, including insulin processing, granular acidification, and ultimately exocytosis. This means that increases in \( \text{Ins}(3,4,5,6)P_4 \) could regulate any or all of these events. Because the increase in \( \text{Ins}(3,4,5,6)P_4 \) is not rapid, its action is likely to influence second rather than first phase insulin release. It may also play a pathologic role during periods of hyperglycemia. This is because \( \text{Ins}(3,4,5,6)P_4 \) production is enhanced when energy metabolism is compromised. In addition, chronic stimulation of PLC that can occur under high glucose would lead to prolonged increases in \( \text{Ins}(3,4,5,6)P_4 \) and, thus, a longer term impairment of insulin exocytosis.

B. Inositol Hexakisphosphate Regulation of Voltage-Gated Ca\(^{2+}\) Channels and Membrane Trafficking

Inositol hexakisphosphate (\( \text{InsP}_6 \)) is the most abundant inositol polyphosphate in a number of cellular systems, ranging from plants to mammalian cells, and it is the major natural organic phosphate in the biosphere (Turner et al., 2002). In this review, we focus particularly on mammalian cells and primarily on pancreatic β-cells, but it is important to recognize the overwhelming presence of \( \text{InsP}_6 \) in biology. We return to this in the next section.

Studies in the pancreatic β cell exposed one of the first clear physiologic roles for \( \text{InsP}_6 \) in mammalian cells (Fig. 5). A more detailed description of the role of \( \text{InsP}_6 \) in β-cells can be found elsewhere (Berggren and Barker, 2008). Here, we describe more briefly the processes in which \( \text{InsP}_6 \) is involved.

When considering functions for \( \text{InsP}_6 \) in the wake of the Ca\(^{2+}\)-mobilizing second messenger, \( \text{Ins}(1,4,5)P_3 \), it was natural to consider Ca\(^{2+}\) regulation as a putative target area, especially because it was a known Ca\(^{2+}\)-chelator (Gersonde and Weiner, 1981). In the β-cell, in keeping with many other neuroendocrine or neuronal cells, mobilization of Ca\(^{2+}\) from intracellular stores plays a more secondary role to influx of Ca\(^{2+}\) through PM-standing, voltage-dependent Ca\(^{2+}\) channels (Yang and Berggren, 2005). In β-cells, these channels are physically coupled to the exocytotic machinery (Yang et al., 1999) and are responsible for providing the Ca\(^{2+}\) that drives the final steps of exocytosis and, thus, insulin secretion (Yang and Berggren, 2005). In an article in Science (Larsson et al., 1997), it was established that \( \text{InsP}_6 \) enhanced L-type \( \text{Ca}^{2+} \) channel activity and that, although other pentakisphosphates could also mediate this effect, they were less potent than was \( \text{InsP}_6 \). Two other studies, one on hippocampal neurons (Yang et al., 2001) and one on vascular smooth muscle cells (Quignard et al., 2003), also showed the enhancing effect of \( \text{InsP}_6 \) on L-type voltage-dependent Ca\(^{2+}\) channels, although in the smooth muscle cells, there was not a marked difference between the potency of \( \text{InsP}_6 \) and that of \( \text{Ins}(1,3,4,5,6)P_5 \). The L-type voltage-dependent Ca\(^{2+}\) channel is controlled through protein phosphorylation; in β-cells, a mechanism of action of \( \text{InsP}_6 \) was to inhibit serine threonine
phosphatase activity, and thus, a likely mechanism of action would be to promote channel activity through increased phosphorylation (Larsson et al., 1997). The studies on hippocampal neurons showed that InsP_6 could regulate channel phosphorylation, not only by inhibition of serine threonine phosphatases, but also by the activation of PKA. This, in turn, was an indirect effect of increasing cAMP by the stimulation of adenylate cyclase activity (Yang et al., 2001). In studies in the β-cell, it was shown that PKC isoforms can also be activated by InsP_6 (Efanov et al., 1997; Hoy et al., 2003). Thus, InsP_6 regulates L-type voltage-dependent Ca^{2+} channel phosphorylation both by the inhibition of serine threonine phosphatases and by the activation of serine threonine kinases, namely PKA and PKC.

Because of the high basal InsP_6 concentration in β-cells (50–60 μM) (Larsson et al., 1997), this inositol phosphate could serve to establish the set point of channel phosphorylation and, thus, its activity. Glucose stimulation further increased InsP_6 global concentrations; this increase was modest (10–15%), but it occurred within the period in which a large increase in [Ca^{2+}]_i can be observed. To have a functional significance, this small increase in InsP_6 concentration needs to occur in a microdomain close to the channel. Although there are precedents for other β-cell channels, such as the K_ATP channel, existing in microdomains, there is no direct evidence that InsP_6 is associated with such a domain. In other cell systems, however, there is evidence for a pool of InsP_6 associating with the plasma membrane (Poyner et al., 1993), which would represent a separate compartment. In such a plasma membrane-associated pool, it is possible that the small changes in InsP_6 could have physiologic significance to adjacent channels. Compartmentalization of InsP_6 is also indicated by recent work showing that the InsP_6 kinase, responsible for its synthesis, can have distinct cellular localizations. These are in the nucleus (Brehm et al., 2007) and in cilia structures (Sarmah et al., 2007).

As we have stated above, the Ca^{2+} channel is tightly coupled to the exocytotic machinery, which suggests that InsP_6, at least indirectly, could be coupled to exocytosis. However, work on neuronal cells had indicated a negative effect of InsP_6 on neurotransmission by binding to the key exocytotic protein synaptojanin (SJ), a PtdIns(4,5)P_2 5-phosphatase. The inhibition of the lipid phosphatase leads to enhanced levels of PtdIns(4,5)P_2, an important element in the endocytotic process. Finally, through IP6K1, InsP_6 is converted to PP-Insp_5 (InsP_5/IP_5), which regulates the cell’s exocytotic capacity at the level of the readily releasable pool of vesicles (RRP).

Fig. 5. Role of higher inositol phosphates in β-cell function. There are many different arenas in which higher inositol polyphosphates operate, particularly InsP_6 (inositol hexakisphosphate) and InsP_7 (5-diphosphoinositol pentakisphosphate, PP-Insp_5). InsP_6 stimulates L-type VDCC activity, increasing phosphorylation of the channel by both inactivating serine threonine protein phosphatases and stimulating serine threonine kinases (PKC and indirectly PKA through activation of adenyl cyclase) (Yang et al., 2001). The enhanced Ca^{2+} influx driven by InsP_6 will also increase exocytosis, but InsP_6 can also enhance secretion more directly through the activation of PKCε. InsP_6 also targets dynamin-mediated endocytosis through a complex process that involves the dephosphorylation of dynamin through calcineurin and InsP_6’s ability to both stimulate PKCε and inhibit synaptojanin (SJ), a PtdIns(4,5)P_2 5-phosphatase. The inhibition of the lipid phosphatase leads to enhanced levels of PtdIns(4,5)P_2, an important element in the endocytotic process. Finally, through IP6K1, InsP_6 is converted to PP-Insp_5 (InsP_5/IP_5), which regulates the cell’s exocytotic capacity at the level of the readily releasable pool of vesicles (RRP).
2003). These electrophysiological studies also revealed another facet of the role of InsP₆ in vesicle trafficking. Initial experiments using the capacitance technique showed that InsP₆ decreased rather than increased exocytosis. However, with use of a technique that simply reports changes in cell surface area, the final measurement was found to be a composite of exo- and endocytosis. In fact, it was revealed that InsP₆ could drive endocytosis more efficiently than could exocytosis (Hoy et al., 2002); thus, when endocytosis was inhibited, InsP₆ exocytotic function was exposed. This led to a more detailed study of the role of InsP₆ in β-cell endocytosis. InsP₆ targets dynamin-mediated endocytosis through a complex process that involves the dephosphorylation of dynamin through calcineurin and the ability of InsP₆ to both stimulate PKC and inhibit synaptotagmin, a PtdIns(4,5)P₂ 5-phosphatase. The inhibition of the lipid phosphatase led to enhanced levels of PtdIns(4,5)P₂, an important element in the endocytotic process (Hoy et al., 2002). These studies expose a novel cross-talk between inositol polyphosphate and lipid metabolism.

C. Novel Roles of InsP₆ Outside the β-Cell

When considering the role of InsP₆ outside the β-cell, a large number of functional roles have been suggested, especially in mammalian cells (Irvine and Schell, 2001; Irvine, 2005). This vast number of roles provoked Shears to entitle a review on the subject “Assessing the Omnipotence of Inositol Hexakisphosphate” (Shears, 2001). In this review, Shears eliminated many of the putative roles on the basis of a rigorous application of logic and a careful examination of the physiologic relevance of the conditions in which InsP₆ was tested. We mainly focus on those roles that were not eliminated by this review. We should clarify that the importance of InsP₆ stems both from its own role and as a platform from which other regulatory molecules are derived. Examples of the latter include the inositol pyrophosphates (Barker et al., 2009b; Shears et al., 2011; Saiardi, 2012) (see section IV) or the Ins(1,2,3)P₃-based inositol polyphosphates with iron-binding properties (Barker et al., 1995; Spiers et al., 1996; Irvine, 2005). In terms of the direct effects of the molecule, two or three different modes of operation for InsP₆ can be identified. First, insights from nutritionists clearly indicate its importance as a metal ion chelator, especially of iron (Zhou and Erdman, 1995). This continues to be the case for human nutrition, although a particular role in intracellular iron metabolism has now been challenged (Torres et al., 2005). Second, InsP₆ acts as an allosteric regulator of specific proteins, and third, in a related but not identical role, it also acts as a structural component of some important enzymes.

The role of InsP₆ as a chelator in iron metabolism and as an antioxidant (Graf et al., 1987) has diminished in the light of a study showing that, in the ionic environment of the cytosol, it exists as a neutral pentavalent Mg²⁺ salt that has no capacity to bind iron (Torres et al., 2005). However, it still may fulfill this role in other cellular contexts in vesicles or other subcellular organelles and more widely in nutrition, in which its abundance and chelating properties are known to cause malnutrition in developing countries (Zhou and Erdman, 1995). Furthermore, the InsP₆ breakdown product Ins(1,2,3)P₃ (Barker et al., 1995) has now been subject to the same rigorous assessment as InsP₆ (Veiga et al., 2009) but, in contrast, remains one of the best candidates in the mammalian cell for both complexing iron and preventing iron-catalyzed free radical cascades, acting therefore as a potent antioxidant. The importance of InsP₆ as an antioxidant then becomes as the reservoir from which Ins(1,2,3)P₃ is derived.

As mentioned in the introductory section, studies on yeast first exposed a fundamental role for InsP₆ in the context of the nucleus. This novel function was the export of mRNA from the nucleus to the cytoplasm (York, 2006). This was established by deletion experiments that systematically removed combinations of plc1, ipk2, and ipk1, the yeast homologs of PLC, inositol polyphosphate multikinase (IPMK or Ipk2), and Ins(1,3,4,5,6)P₅ 2-kinase (or Ipk1), respectively (Fig. 1). InsP₆ lies at the end point of the pathway controlled by these enzymes (Fig. 1) (York et al., 1999). Subsequent studies have revealed that InsP₆ specifically binds to the C-terminal of Gle1, a nuclear-pore associated protein (Folkmann et al., 2011). This interaction leads to increased activation of Dbp5, a highly-conserved ATPase, which is thought to use its ATPase activity to remodel RNA. The constitutive activity of Dbp5 is not sufficient to drive mRNA export; this apparently requires the InsP₆/Gle1 complex. More recently, the InsP₆/Gle1 activation of Dbp5 has been shown to operate during another aspect of mRNA processing, namely, in translational termination (Alcazar-Roman et al., 2010).

Continuing on this mRNA-based theme, a third aspect of the mode of operation of InsP₆ occurs in mRNA editing. During the determination of the crystal structure of the RNA editing enzyme ADAR2, InsP₆ was found to be present and tightly bound to the enzyme. Furthermore, InsP₆ was a necessary cofactor for enzyme activity (Macbeth et al., 2005). This discovery is reminiscent of an earlier study in which InsP₆ was identified as a factor required for the activity of DNA-dependent protein kinase (DNA-PK) (Hanakahi et al., 2000), although a later work (Ma and Lieber, 2002) indicated that subunits of this kinase complex (Ku70/80) were the actual binding sites of InsP₆. Of interest, there is some resemblance between the binding domain proffered by the Ku70/80 complex and ADAR2. It is possible, as other structures are resolved, that InsP₆ may arise again in interesting but
maintains genomic stability and cell viability. This is caused by genotoxic agents. This repair process is a process that repairs double DNA strand breaks, nonhomologous end joining (Cheung et al., 2008), mediates through its binding to the DNA-PK complex, and this was suggested to occur through synaptotagmin (Llinás et al., 1994). The new data show, at the molecular level, that InsP₆ dose-dependently inhibits hippocampal autaptic excitatory postsynaptic currents. It does this by the inhibition of the synaptotagmin-1 C2B domain-mediated fusion by interacting with the synaptotagmin Ca²⁺-binding sites. Thus, the elevation of InsP₆ levels in activated neurons mediates a unique negative feedback signal that controls hippocampal excitatory transmission.

IV. Inositol Pyrophosphates

One of the important and exciting new frontiers in the inositol polyphosphate field has been the growing revelation that inositol pyrophosphates serve as critical regulators in many cell systems (Barker et al., 2009b; Chakraborty et al., 2011; Shears et al., 2011; Saiardi, 2012). Again, the pancreatic β-cell has provided an important platform for discovering the physiologic relevance of this novel inositiide family (Barker et al., 2009a). The inositol pyrophosphates, or more chemically accurate, the diphosphoinositol phosphates, were formally identified in two separate studies (Menniti et al., 1993; Stephens et al., 1993). Materials with similar charge had been observed previously in HPLC traces of inositol phosphate extracts from several mammalian cells (Barker et al., 2009b). In β-cells, for example, a pyrophosphate was seen in an HPLC trace showing the separation of β-cell inositol phosphates in an article in Science (Larsson et al., 1997) a decade before its physiologic relevance was understood. A more detailed discussion concerning the chemical nature of inositol pyrophosphates can be found in several recent reviews (Barker et al., 2009b; Shears, 2009; Saiardi, 2012). Their configurations are based on two separate parent structures: inositol 1,3,4,5,6-pentakisphosphate [Ins(1,3,4,5,6)P₅] and InsP₆. Furthermore, although there appears to be some overlap of function between the two families, they can act independently in disparate areas of cellular regulation (e.g., telomere length) (Saiardi et al., 2005; York, 2006) and Akt/PKB regulation (Chakraborty et al., 2010), respectively. In nature, the diester pyrophosphate has been identified in several different positions on the inositol ring (Barker et al., 2009b), but in the context of this review, we focus on the species found in mammalian cells. In this case, the phosphates at the 5 or 1 positions of the inositol ring are further phosphorylated. The 1 and 3 positions are enantiomeric and, thus, difficult to assign structurally; only very recently has the actual structure been assigned to the 1 position (Wang et al., 2012). There is another opportunity for confusion from the existing literature, because the original structures were given the preliminary assignment of pyrophosphorylation in the 4 or 6 positions, not the 1 or 3 positions (Mulugu et al., 2007). This was then corrected by a consortium that included the original authors (Lin et al., 2009). Therefore, in the original articles (Lee et al., 2007; Mulugu et al., 2007), the proposed structures are incorrect. There are diphosphoinositol and bisdiphosphoinositol species of both parent molecules. The diphosphopho- and bisdiphosphoderivatives of InsP₆ have been colloquially called IP₇/InsP₇ and IP₈/InsP₈, based on the number of phosphates possessed by the ring and again are found in many prominent publications. However, this choice of nomenclature can introduce confusion at two levels. First, it is possible to also call Ins(1,3,4,5,6)P₅ pyrophosphate derivatives InsP₇ even if they have an entirely different structure. Second, it is possible, at least in overexpression studies, for inositol hexakisphosphate kinases (IP6Ks) to sequentially phosphorylate the Ins(1,3,4,5,6)P₅ or InsP₆ structure at the same position on the inositol ring, creating strings of phosphate groups (Draskovic et al., 2008) that could also be legitimately called InsP₇ and InsP₈. Therefore, in this review, we abbreviate these molecules by a more chemically accurate shorthand. However, the reader must bear in mind that journals have fairly arbitrarily adopted the terms IP₇ and IP₈ to mean the diphospho- and bis-diphosphoderivatives of InsP₆. The use of this abbreviation harks back to the now archaic precedent of referring to Ins(1,4,5)P₃ as IP₃, even though there are multiple inositol trisphosphates existing in mammalian cells, with at least three having completely distinct functions (Barker et al., 1995; Irvine, 2005; Shears et al., 2012).

The two isomers of InsP₇ that have been found in mammalian cells, 5-diphosphoinositol pentakisphosphate (5-PP-InsP₅) and 1-diphosphoinositol pentakisphosphate (1-PP-InsP₅), are formed by two classes of kinases, namely the IP6Ks and the diphosphoinositol pentakisphosphate 1-kinases (PPI5K/VIPs), respectively (Fig. 1) (Barker et al., 2009b; Chakraborty et al., 2011; Shears et al., 2011; Saiardi, 2012). In most cells examined, the 5-isomer is the dominant form. The bis-diphospho-derivative (or InsP₇/IP₇) is much less abundant and can be formed by the phosphorylation of
either (5-PP-InsP₅) by PPIP5Ks or from (1-PP-InsP₅) by IP6Ks (Barker et al., 2009b; Chakraborty et al., 2011; Shears et al., 2011; Saiardi, 2012). The IP6Ks exist as three forms (IP6K1,2,3), and the PPIP5K/VIPs exist as two (PPIP5K/VIP1,2). It has recently been claimed that concentrations of 1,5-Bis-diphosphoinositol tetrakisphosphate ((1,5)-[PP]₂-InsP₄) have been underestimated because of its acid liability during acid-based extraction and HPLC separation techniques (Losito et al., 2009). If this is the case, the actual cellular concentration of (1,5)-[PP]₂-InsP₄ could be significantly higher than studies have thus far indicated. When considering the IP6Ks, of importance, they exert their influences both through their catalytic activity and through specific protein-protein interactions (Barker et al., 2009b). Thus, IP6K1 is involved in exocytosis through both catalytic activity and protein-protein interactions, and the same is true for the involvement of IP6K2 in apoptosis (Barker et al., 2009b).

(1,5)-[PP]₂-InsP₄ is currently not described in the pancreatic β-cell, but our unpublished data (C. Illies, C. J. Barker, P.-O. Berggren) suggest that a material exists that is consistent in polarity with an inositol phosphate possessing 8 phosphate groups. However, it is found at significantly lower concentrations than 5-PP-InsP₅. This may reflect the acid-lability of (1,5)-[PP]₂-InsP₄ that was noted in the last section. However, neutrally extracting pancreatic β-cells does not dramatically enhance the yield of this material (C. J. Barker, C. Illies, and P.-O. Berggren, unpublished observations). It is also unclear which PPIP5Ks exist in β-cells. More is known about the IP6Ks and their diphosphoinositol products. As expected from other studies of mammalian cells, 5-PP-InsP₅ is the dominant isomer in β-cells (Illies et al., 2007). However, its concentration is unusually high, reflected both by the fact that it exists at around 6–10% of the InsP₆ peak, whereas in other mammalian cells, it is closer to 1–3% (Shears, 2009), and that estimates of its concentration in insulin-secreting cells is ~6 µM (Illies et al., 2007), again substantially higher than found in other mammalian cells, generally ~1 µM (Shears, 2009; Saiardi, 2012).

A. The Mechanism of Action of Pyrophosphates

Studies performed in the past eight years have revealed that inositol pyrophosphates may exert their influence by two modes of action, one based on classic allosteric interactions and the second by a novel phosphate transfer reaction. In both cases, aspects of the work have been challenged, although there is clearly enough data now to assert that the suggested two modes of action need to be taken seriously.

Because of the doubts now expressed on the physiologic relevance of the binding of inositol pyrophosphates to mammalian adapter protein modules (Shears, 2001; Shears et al., 2012) (see section IV.B), the first clear example of allosteric modulation exerted by inositol pyrophosphates comes from yeast (Lee et al., 2007). However, this inositol pyrophosphate is not the dominant inositol 5-PP-InsP₅ in mammalian cells (formed by the IP6Ks), but 1-PP-InsP₅, formed via PPIP5Ks and is a less abundant variant. It is important to reinforce here an earlier remark. In the studies on yeast, the novel PP-InsP₅ isomer is referred to as being one of the 4/6-PP-InsP₅ enantiomeric pair (Lee et al., 2007). This is incorrect. In this review, we use the correct structural allocation. 1-PP-InsP₅ is associated with the regulation of the Pho complex, an assembly of cyclin-related proteins consisting of a kinase, a cyclin, and an inhibitory protein, Pho81. This complex is an established regulator of yeast phosphate homeostasis. 1-PP-InsP₅ strengthens the inhibitory action of Pho81. The interpretation of some of these data has been challenged, especially in the integration between 1-PP-InsP₅ concentrations and changes in phosphate levels (Onnebo and Saiardi, 2007). Nonetheless, the binding shows specificity, because the common 5-PP-InsP₅ does not significantly bind, and neither does InsP₆.

Within the past couple of years, another example of inositol pyrophosphate-mediated regulation has been proposed. It has been suggested that 5-PP-InsP₅ acts to inhibit the key regulatory protein Akt/PKB (Chakraborty et al., 2010). This interaction must be subject to careful scrutiny, because other inositol phosphates existing at much high concentrations than the pyrophosphates have also been suggested to inhibit Akt/PKB [e.g., Ins(1,3,4,5,6)P₅] (Piccolo et al., 2004). Furthermore, even the binding of 5-PP-InsP₅ to this protein has been disputed (Downes et al., 2005). However, the authors of this new work are not just proposing a direct effect on Akt/PKB, but a more subtle interference of the interaction between Akt/PKB and the regulatory protein PDK1 (Chakraborty et al., 2010). We have debated these complexities in more detail elsewhere (Barker et al., 2009b). It is possible to combine the previous negative data with the current positive data when considering the different conditions in which the binding studies were performed. In summary, the central issue is that the pre-binding of PtdIns(3,4,5)P₃ to Akt/PKB prevents 5-PP-InsP₅ binding. Therefore, in studies such as those performed by the Downes laboratory (Downes et al., 2005), in which displacement of phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P₃] was used to quantify 5-PP-InsP₅ binding, 5-PP-InsP₅ appears to bind Akt/PKB poorly. However, in the studies from the Snyder group and others, in which PtdIns(3,4,5)P₃ is not prebound, 5-PP-InsP₅ binds and inhibits Akt/PKB (Barker et al., 2009b). Therefore, we remain open to the possibility that these initial studies will be supported by future experimental investigations.
The background to the proposition that 5-PP-InsP₅ regulates Akt/PKB signaling arose from a study of the phenotype of a mouse in which IP6K1 has been globally knocked out (Bhandari et al., 2008). This mouse exhibits a striking increase in insulin sensitivity and a resistance to obesity, which is clearly consistent with enhanced Akt/PKB signaling, but other explanations are also possible (see below) (Chakraborty et al., 2010). Moreover, it is not clear whether this regulation is general to all Akt/PKB isoforms or specific to one. Verification of this is important, especially in the pancreatic β-cell, in which different Akt/PKB isoforms exert control over distinct signaling pathways (Buzzatti et al., 2010; Leibiger et al., 2010). We shall return to this in the context of the role of InsP₇ in exocytosis. Of significance, this inhibition is specific to the abundant 5-InsP₇ isoform (the 1-PP-InsP₅ isomer is ineffective) and, thus, shows stereo-selectivity, an important prerequisite for allosteic regulation. It also highlights a role for IP6Ks rather than PPIP5Ks in these events.

The second method of action of inositol pyrophosphates is through a so-called pyrophosphorylation reaction. In this case, a high-energy phosphate from the inositol pyrophosphate, rather than ATP, is used to posttranslationally modify a specific subset of proteins that are not regulated by the classic ATP-dependent phosphate donation. Initially, this was suggested to be a kinase-independent event (Saiardi et al., 2004), but now it is clear that prephosphorylation by CK2 is required (Bhandari et al., 2007). Therefore, the phosphorylation by pyrophosphates is not a classic phosphorylation but a pyrophosphorylation on an already phosphorylated serine residue (Bhandari et al., 2007). It is widely accepted that this pyrophosphorylation can occur in an in vitro setting, but its role in the intact cellular environment is more contentious (Shears et al., 2011). Hurdles that have to be overcome include the fact that InsP₆, which is generally one of the most abundant inositol polyphosphates in mammalian cells, inhibits the reaction. The current defense of this discrepancy has involved cellular compartmentalization, and although this may not be accurate, the compartmentalization of the Ins(1,3,4,5,6)P₅ 2-kinase, exclusively responsible for InsP₆ synthesis and that we have mentioned in the section on InsP₆, gives some substance to segregation (Brehm et al., 2007; Sarmah and Wente, 2009).

Since the original articles describing and refining the nature of the pyrophosphorylation (Saiardi et al., 2004; Bhandari et al., 2007), there have been a recent study that highlights the functional involvement of this novel posttranslational modification in an important cellular process and another study that questions it (Azevedo et al., 2009). On the positive side, pyrophosphorylation of the β3A subunit of the AP3 complex, when bound to kinesin, promotes a dissociation that prevents AP3-mediated HIV Gag protein trafficking (Azevedo et al., 2009). In this latter study, a back phosphorylation protocol was used to better verify the relevance of the pyrophosphorylation in intact cells. However, other interpretations are possible (Shears et al., 2011). On the negative side, another laboratory, using an admittedly indirect method, were not able to find an association between pyrophosphorylation and function (Yang et al., 2008). This study relates to the human homolog of Nsr1 or nucleolin, which is pyrophosphorylated in yeast (Saiardi et al., 2004). The authors were unable to correlate assumed pyrophosphorylation of this protein with levels of inositol pyrophosphates that might be predicted to influence it (Yang et al., 2008), although it is possible that, in the different biologic systems (yeast and mammalian), other factors come into play. For example, the authors studying the human protein used the same kind of ploy that had been exploited in the yeast system, but without being able to establish a clear linkage (Yang et al., 2008). For this novel posttranslational modification to gain broader acceptance, further independent verification would be helpful. Because data on β-cell exocytosis, discussed in the next section, seemed not to select between PP-InsP₅ isoforms (Illies et al., 2007), there was an indirect indication that pyrophosphorylation may play a role in these cells. The reason for this is that pyrophosphorylation can be performed by a variety of inositol pyrophosphates without much selectivity between them. Although we have looked carefully, we have yet to produce convincing evidence that pyrophosphorylation occurs in β-cells (C. Illies, P.-O. Berggren, and C. J. Barker, unpublished results). However, of importance, the current strategy to identify new pyrophosphorylated proteins is through a genomic rather than proteomic approaches (Werner et al., 2010). This tactic may be driven by the inability of current mass spectrometry methods to reliably identify the pyrophosphorylated motif in a protein (Shears et al., 2011).

B. Inositol Pyrophosphates as Regulators of Exocytosis

The observation of the higher levels of 5-PP-InsP₅ in insulin-secreting cells, mentioned in the introduction to Section IV, was also confirmed in primary islets and β-cells (Illies et al., 2007), which paved the way for a detailed investigation of a potential role of this pyrophosphate in the β-cell. When we consider a role for inositol pyrophosphates in pancreatic β-cells, we turn to exocytosis. There has been an indication from the work of Snyder and colleagues that one of the kinases involved in 5-PP-InsP₅ production, IP6K1, is involved in neuronal exocytosis (Luo et al., 2001). However, this was independent of any catalytic activity and, thus, not related to the generation of inositol pyrophosphates. Earlier studies have suggested the involvement of InsP₇ in vesicle trafficking, related to
its high affinity for subunits of the AP2 and AP180 adaptor proteins associated with the formation of clathrin coated vesicles, but as we have discussed above, it is now thought to be unlikely that this has a physiologic context (Shears, 2001). Deletion of IP6Ks in yeast led to disruption of intracellular membrane compartments. However, in mammalian cells, inositol pyrophosphates had no established track record in influencing vesicle trafficking or the resultant exocytotic events.

It was established, through electrophysiological experiments measuring membrane capacitance, that 5-PP-InsP$_5$ was important for the filling of the RRP of vesicles and, thus, controlled the exocytotic potential of the β-cell (Illies et al., 2007). The effect of InsP$_7$ was dose-dependent in the physiologic concentration range of the pyrophosphate found in mammalian cells. Although overexpression of either of the IP6Ks (IP6K1 and 2) found in β-cells increased the RRP, only the siRNA knockdown of IP6K1 had any effect on exocytosis. These data hint at a spatial segregation in 5-PP-InsP$_5$ production, which, as we have indicated earlier, has several precedents in β-cell stimulus-secretion coupling. It has previously been shown that InsP$_6$ can promote exocytosis in pancreatic β-cells (Efanov et al., 1997; Hey et al., 2003), but the effect was not restricted to the RRP-like 5-PP-InsP$_5$ (Illies et al., 2007). Thus, a clear delineation can be made between the effects of InsP$_6$ and 5-PP-InsP$_5$ on exocytosis, and this suggests that they act through independent mechanisms.

From a physiologic perspective, the RRP has been suggested to reflect the first phase of insulin secretion after glucose stimulation (Rorsman and Renstrom, 2003). A caveat needs to be added about the RRP pool, because it is an empirical definition based on insights from electrophysiology. Recent insights using alternative techniques may give a different picture. The most prominent new approach is based on the total internal reflection microscopy technique, which exploits an evanescent wave to selectively follow events at or just below the plasma membrane. This procedure has suggested that the electrophysiological RRP, based on direct membrane depolarization, causes the fusion of a pool of vesicles that hardly overlaps with the pool of vesicles that glucose stimulation alone can drive to fusion (Seino et al., 2011). In the case of the data published in the article in Science on the effect of 5-PP-InsP on the RRP (Illies et al., 2007), both electrophysiological and indirect measurements of insulin secretion were used to draw the conclusions. Furthermore, we have recently developed a direct dynamic insulin release assay, and silencing IP6K1 in this system reduces the first phase of insulin release (G. C. Gaboardi, P. O. Berggren, and C. J. Barker, unpublished data). Because this phase is thought to reflect the liberation of the RRP, we can conclude that 5-PP-InsP$_5$ also regulates the glucose-induced RRP.

The report that 5-PP-InsP$_5$ and, thus, IP6K1 is important for insulin secretion is backed by two further pieces of evidence. First, a genetic linkage study in a Japanese family with T2D found a putative disruption of the IP6K1 gene (Kamimura et al., 2004), and second, in an article by Snyder and colleagues, in which they made a general knockout of IP6K1 in a mouse, showed that it had decreased insulin in the blood, consistent with our studies on β-cell insulin secretion (Bhandari et al., 2008). The IP6K1 knockout mouse did, however, expose a paradox that may also have a bearing on pancreatic β-cell regulation by pyrophosphates.

Despite the lower level of insulin secretion, the IP6K1 knockout mouse was not diabetic and responded normally to a glucose tolerance test (Bhandari et al., 2008). The explanation for this, as suggested above, was exposed after additional studies (Chakraborty et al., 2010) that revealed that Akt/PKB signaling was enhanced, and this leads naturally to an increased insulin sensitivity. This improved sensitivity was more than able to compensate for the β-cell defect. The mouse is also resistant to the effects of a high-fat diet and is thus a supermouse; however, the actual molecular phenotype at the level of the 5-PP-InsP$_5$/Akt/PKB interaction needs careful consideration. This stems from the fact that there is at least one alternative molecular explanation for the enhanced Akt/PKB signaling resulting from the elimination of IP6K and, second, that there is doubt in the literature regarding the 5-PP-InsP$_5$/Akt binding, both of which we have discussed above.

The first caveat arises from a still unexplained phenomenon that is manifested when silencing IP6K in yeast (Saiardi et al., 2000) or in pancreatic β-cells (Barker et al., 2009b; C. J. Barker, C. Illies, and F.-O. Berggren, unpublished observations). This silencing also reduces the level of Ins(1,3,4,5,6)P$_5$. The effect in yeast is more pronounced, but this may reflect the fact that there is only one IP6K in this organism. Of course, it is possible that it is not Ins(1,3,4,5,6)P$_5$ that is being reduced, but a yet unidentified InsP$_7$-based pyrophosphate with the same elution properties. Snyder and colleagues report that this reduced level of the Ins(1,3,4,5,6)P$_5$ peak is not observed in either brain or embryonic fibroblasts of IP6K1 knockout mice; indeed, there is an increase of Ins(1,3,4,5,6)P$_5$ in IP6K1 knockout brain (A. Chakraborty and S.H. Snyder, unpublished observations). This suggests that lowering of Ins(1,3,4,5,6)P$_5$ concentrations after IP6K knockout may be a phenomenon restricted to β-cells. We raise this issue, because Ins(1,3,4,5,6)P$_5$ is also a known inhibitor of Akt/PKB (Piccolo et al., 2004) (see above), and loss of Ins(1,3,4,5,6)P$_5$ may produce a phenotype similar as that produced by loss of PP-InsP$_5$. Another concern with this mouse model is the reported regulation of 5-PP-InsP$_5$ by insulin. We have
not seen this, at least not in short-term experiments. The effects that are reported are from studies on dividing cells that have been serum starved. Under these conditions, cells can lock in mid G1, and addition of insulin probably releases them from this lock. Because it has also been demonstrated that PP-InsP₅ concentrations increase in the cell cycle during the same time frame (Barker et al., 2004), the changes may reflect cell cycle changes rather than the kind of regulation that would be observed in the mature β-cell, with its low rate of cell division. In fact, if cells are held in a serum-starved state, they can rapidly enter cell cycle again, after serum is re-administered; we have observed (C. J. Barker, unpublished data) that, under these conditions, PP-InsP₅ increases fairly rapidly. However, unpublished data from A. Chakraborty and S. H. Snyder (personal communication) suggest that even short (2 hours) serum starvation protocols can reduce PP-InsP₅ levels. Subsequent addition of growth factors seems to then be able to increase PP-InsP₅. With such a short starvation protocol, changes in 5-PP-InsP₅ may not be linked to specific cell cycle–related changes.

Whatever the final outcome of these deliberations, in terms of β-cell regulation, the possibility that IP6Ks, directly or indirectly, are also modulating Akt/PKB, is an important one. Additional studies by the Snyder and colleagues (Prasad et al., 2011) have indicated an enhanced Akt/PKB signaling in neutrophils from IP6K1 knockout mice, reinforcing a likely in vivo role and its regulation, the possibility that IP6Ks, including IP6K1, are also sensitive to ATP:ADP increases. This possibility is likely, because IP6Ks, including IP6K1, have an unusually high (mM) Kₘ for ATP (Voglmaier et al., 1996; Saiardi et al., 2000). Therefore, the enzymes could be subject to regulation by prevailing millimolar cellular ATP concentrations. Shears, also noting the kinetic properties of IP6Ks with regard to ATP, has

C. Inositol Hexakisphosphate Kinase as a Metabolic Sensor with a Role in T2D

Glucose-stimulated insulin secretion is biphasic in mammals, including humans. One of the key early defects in T2D is a blunted first phase of secretion (Ahren, 2005). As we have discussed above, the RRP is the major contributor to the first phase of insulin release and 5-PP-InsP₅ is a quantitatively significant regulator of the RRP. These fundamental relationships raise the question of whether 5-PP-InsP₅ and/or IP6K1, the kinase that produces it, have any role in the disease phenotype and its progression. One indication that this might be the case is to consider possible genetic evidence linking IP6K1 to T2D. As we have mentioned above, there was a single report describing a Japanese family in which a putative disruption in the IP6K1 gene was proposed to be linked to the disease (Kamimura et al., 2004). However, T2D is a polygenetic disease with many potential dysfunctional genes, and thus, this single case, although indicating a proof of principle, is not one in which a wider involvement of IP6K1 in T2D can be built. Perhaps a more insightful question is how the 5-PP-InsP₅-generated RRP will be affected in a diabetic milieu. This question leads to the discussion of two fundamental properties of IP6K1 that may make it vulnerable in T2D and that are shown in Fig. 6 and discussed below.

To set these properties in context, it is important to re-emphasize the tight connection between glucose metabolism, changes in ATP:ADP ratios, and the drive to stimulate insulin release that occurs in pancreatic β-cells. In summary, in the β-cell, increases in the ATP:ADP ratio, instituted by glucose metabolism, leads to closure of Kₐ₅ channels, depolarization of the β-cell and influx of Ca²⁺ through voltage-dependent Ca²⁺ channels, which then drives the final stages of insulin release. Thus, changes in ATP:ADP are integrated into insulin secretion, via the Kₐ₅ channel (Ashcroft and Rorsman, 1990). There are, however, other examples in which changes in the ATP:ADP ratio are coupled to exocytosis. One of these is phosphatidylinositol 4 kinase, which can sense changes in the ATP:ADP ratio (Olsen et al., 2003). An increase in the ATP:ADP ratio after glucose stimulation leads to its activation, a concomitant increase in the production of PtdIns(4,5)P₂, and the promotion of secretion. With these precedents in mind, it is possible that IP6Ks could also be sensitive to ATP:ADP increases. This possibility is likely, because IP6Ks, including IP6K1, have an unusually high (mM) Kₘ for ATP (Voglmaier et al., 1996; Saiardi et al., 2000). Therefore, the enzymes could be subject to regulation by prevailing millimolar cellular ATP concentrations. Shears, also noting the kinetic properties of IP6Ks with regard to ATP, has
already proposed that they may be metabolic sensors (Shears, 2009). The next important question is, can we connect this ATP:ADP sensitivity of the IP6K to diabetes? Disturbances of ATP and energy production in pancreatic β-cells is an established phenomenon in diabetic models (Ma et al., 2012), and as we have stated in the introduction, the initiation of glucose-induced insulin secretion is dependent on changes in the ATP:ADP ratio. 5-PP-InsP₅ levels seem to be exquisitely sensitive to reductions in cellular ATP levels, even more so than the lipids (Nagel et al., 2010). Thus, there could be a direct coupling of disrupted energy metabolism to a reduced activity of IP6K1. The consequence of this would be a reduction in the RRP and a subsequent diminishing of the first phase of insulin secretion.

The second intrinsic property of IP6K1 pertaining to diabetes is that it is sensitive to oxidants and that oxidant stress leads to its depletion (Onnebo and Saiardi, 2009). The sensitivity to oxidants is a direct one based on a vulnerable cysteine in the active site of the enzyme. Oxidative stress is a consequence of the hyperglycemia associated with both types of diabetes. In T2D, in which the β-cell still survives but malfunctions, oxidative stress continues to play an important role in the death of the cells (Rosen et al., 2001).

In summary, IP6K1 may represent a novel link between known metabolic defects in β-cell stimulus-secretion coupling and the diabetic phenotype exhibited by the cell. Therefore, the enhancement of IP6K1 activity under glucose-stimulating conditions could represent an important new concept in diabetes treatment, because it pertains to the preservation of β-cell function.

D. Insights of Inositol Pyrophosphate Function from Other Cell Systems

Although we have covered many of the important aspects of inositol pyrophosphates in the preceding sections, some topics, principally because there is no extant data coming from β-cells, have been ignored. Therefore, in this section, we focus on some of these neglected areas, because we believe that they are likely to be important in our future understanding of the physiology and pathophysiology of the β-cell, especially during the stress associated with diabetes. The first theme is the role of inositol pyrophosphates in apoptosis. In this context, we need to consider the IP6K2 isoform.

As we have suggested above, IP6K2 is involved in apoptosis, both because of its catalytic activity and, thus, its ability to generate inositol pyrophosphates, presumably 5-PP-InsP₅, and its noncatalytic activity (Barker et al., 2009b). This area is confused by the fact that two separate groups have described very different redistributions of IP6K2 during apoptosis. The explanation for these discrepancies may lie in the different pathways by which apoptosis is being induced or other cell type–specific phenomena. Essentially, the initial reports suggested that IP6K2 translocated to the nucleus in IFN-β- (and later γ-radiation) induced apoptosis (Morrison et al., 2001, 2002), whereas studies by another group suggest a normal nuclear localization, followed by a translocation to bax-positive mitochondria after pro-apoptotic stimuli, such as cytotoxic stress (Nagata et al., 2005). In trying to sort out the discrepancy between the work of these two groups, the first thing to note is that IP6K2 has a nuclear localization signal and, thus, has the intrinsic property to localize to the nucleus. This would thus support the idea that IP6K2 can travel to the nucleus. However, it does not exclude the possibility of the second case: that is, after it is in the nucleus, it could leave under the appropriate stimulation. Therefore, it is possible that both sets of data are correct but represent different scenarios occurring in different cell types in which IP6K2 is or is not nuclear resident. One possible mechanism by which a protein with a nuclear localization signal remains resident in the cytoplasm is if it is complexed to another protein that masks its localization signal or in some other way restricts its access to the nucleus. In this regard, it is interesting that IP6K2 has been shown to bind to the heat shock protein HSP90 (Chakraborty et al., 2008). This association occurs in the cytosol and masks the catalytic activity of the IP6K2. Dissociation of IP6K2 from HSP90 occurs during cytotoxic insults, including those generated by anticancer drugs (Chakraborty et al., 2008). It is possible that cytosolic IP6K2 is invisible to antibodies until it is released from its partner, which would be one way of combining the two sets of disparate data. Very recently, insights from studying IP6K2 in lymphoblast cells from patients with Huntington’s disease suggest that IP6K2 is in the nucleus under normal conditions, but in the cytosol during the disease state (Nagata et al., 2011). We return to this disease model later, but these new data allow us to speculate that the location of an active IP6K2 in the cytosol, and not the nucleus, may reflect a pathologic phenotype of the ovarian cancer cells used in the original studies (Morrison et al., 2001, 2002) and may be another explanation for the discrepancy between the data from the two groups.

When we considered IP6K-silencing experiments with respect to exocytosis, it was clear that only the IP6K1 had the endogenous capacity to mediate the enhancement of 5-PP-InsP₅ of the RRP, although flooding of the cell with this pyrophosphate by overexpression of any IP6K can also mediate this effect (Illies et al., 2007). This thus raised the issue of compartmentalization. Of interest, when we consider experiments that involve the IP6K2 catalytic activity in driving apoptosis, a similar phenomenon is apparent. That is, overexpression of any IP6K can promote
apoptosis, but only silencing of IP6K2 can prevent apoptosis, at least that driven by cytotoxic stress (Nagata et al., 2005). Thus, here too, there is a suggestion of apparent compartmentalization. Another strong indication of the compartmentalization of IP6Ks comes from the phenotypes of the knockout mice in which IP6K1 and IP6K2, respectively, are deleted. We consider these phenotypes in more detail now, because they give an important understanding to the broader role of IP6K2 in cell and whole animal physiology (Bhandari et al., 2008; Morrison et al., 2009).

As we have described in Section IV.B, the phenotype of the IP6K1 knockout mouse is a reduced plasma insulin level (Bhandari et al., 2008), which probably reflects reduced insulin secretion (Illies et al., 2007). It also has a reduced body mass, and the males are infertile. In contrast to the IP6K1 knockout mouse, the IP6K2-knockout mouse has no reduction in plasma insulin levels or body mass; moreover, it is fertile (Morrison et al., 2009). Although these findings reinforce the unique contribution of location of the individual IP6Ks, we need to bear in mind that both kinases also exert effects independently of their catalytic activity. Nonetheless, it is apparent that the generation of inositol pyrophosphates is important in both apoptosis and exocytosis and that this can be independent of any specific protein-protein interactions (Nagata et al., 2005; Illies et al., 2007). Focusing on the unique phenotype of the IP6K2 knockout mouse, the authors were able to demonstrate an increased generation of oral- and esophageal-based squamous cell carcinomas when animals were given a carcinogen in their drinking water (Morrison et al., 2009). Furthermore, fibroblasts from the knockout animals displayed insensitivity to the antiproliferative effect of interferon β, indicating that the phenotypes observed in the previous experiments based on cultured cells can be observed in vivo. Although there is no obvious aspect of the IP6K2 knockout phenotype that suggests an important role of this enzyme in β-cells (i.e., unlike IP6K1, it does not contribute noticeably to insulin secretion), its role in apoptosis should not be neglected. This is because apoptosis in the pancreatic β-cell underscores both T1D and T2D (Thomas et al., 2009).

As we have mentioned above, a new study advocates a correlation between cellular distribution of IP6K2 in lymphoblasts and the HD phenotype (Nagata et al., 2011). In fact, the suggestion is that the residence of IP6K2 in the cytosol, which leads to an increase in basal 5-PP-InsP5, drives the autophagy phenotype seen in this disease. This is an important observation; however, it raises an issue that may also be significant in diabetes. An early article by the same group had shown that IP6K2 overexpression promotes autophagy (Nagata et al., 2010), and this was based on the ability to generate inositol pyrophosphates. Autophagy is an important catabolic process maintaining proper organelle homeostasis in cells (Szumiel, 2011). Recent work has shown that autophagy is important in the maintenance of proper growth and function of β-cells, indicated by the fact that islets from mice in which autophagy has been disrupted in the β-cells, show reduced β-cell mass and compromised insulin secretion (Hur et al., 2010). Because autophagy is also upregulated in insulin resistance, its induction may also represent a defensive strategy against T2D. IP6K2 is likely to be similarly vulnerable to disruption by ATP/ADP changes and oxidative stress as IP6K1, therefore returning to the model that we proposed in section IV.B; it is obvious that a compromised 5-PP-InsP5 generation—this time, as a consequence of IP6K2 inactivation—could mediate a reduced autophagy in pancreatic β-cells. This indicates another important process in which compromised inositol pyrophosphate generation could act to promote β-cell dysregulation in T2D. It is possible that the elevated levels of 5-PP-InsP5 in β-cells do not just underscore the maintenance of the exocytotic potential, but also may help sustain the basal autophagy required for effective β-cell function. One caveat that needs to be raised is that IP6K2 also mediates apoptosis; thus, there are both positive and negative aspects to its activation. Under normal circumstances, the level of 5-PP-InsP5 that is generated may not be sufficient to be proapoptotic but can still mediate a positive effect on autophagy, thus protecting the β-cell; however, when the cell experiences more severe stress, the proapoptotic dimension may come to the fore.

V. The Future of Inositol Polyphosphate Regulation in β-Cells and Beyond

In this concluding section, we consolidate our examination of the role of inositol phosphates in the β-cell by suggesting important areas for future development. We believe that the synergism between inositol phosphates and β-cell physiology will continue to expand into an exciting future, especially because current evidence suggests that this family of molecules is likely to underscore several aspects of β-cell pathology in diabetes. Because the β-cell is a paradigm on which a broader understanding of cellular biology can be built, we also highlight areas of interest in the β-cells that will have a wider impact in cell biology.

First, of note (Barker and Berggren, 2010), the most neglected aspect of inositol research, in terms of both pancreatic β-cells and cell biology in general, is the study of the basic building block, myo-inositol. Everything else originates from this molecular structure. Inevitably, therefore, inositol is the gatekeeper to the inositolite family. Its relevance in pancreatic β-cells was underscored by earlier studies on β-cell stimulus-secretion coupling. In these studies, it was reported
that inositol was an important factor in optimizing the cell’s secretory response to glucose (Clements and Rhoten, 1976; Pace and Clements, 1981). The number and variety of molecules based on this simple building structure and their diverse roles in cellular regulation are significant (Fig. 1); therefore, the inositol depletion that is seen in diabetes (Greene et al., 1989) will affect not only GPCR signaling, as was originally supposed, but also many critical areas of cellular regulation.

Although studies in other cell systems have indicated that the concentration of signaling molecules, such as Ins(1,4,5)P3, only appears to be affected by the severe inositol depletion caused by, for example, lithium treatment, other inositol phosphates not directly related to cellular signaling, such as Ins(1,2,3)P3, are more sensitive to prevailing inositol concentrations (Barker et al., 1995). This latter observation may be important, because Ins(1,2,3)P3 has antioxidant properties (Speiers et al., 1996; Veiga et al., 2009). Thus, the inositol depletion observed in diabetes will lead to the suppression of an important protective antioxidant in a number of tissues, including pancreatic β-cells. This is particularly relevant with the recent advocacy that the generation of free-radicals takes center-stage in our current understanding of diabetic complications (Brownlee, 2005).

After inositol, we turn to Ins(1,4,5)P3, the forerunner of most of the inositol polyphosphates that we observe in β cells at present. An important question is whether such an established player can contribute more to the future aspects of β-cell research. As we have highlighted earlier, the interrelationships among Ins(1,4,5)P3, ER Ca2+ stores, and voltage-dependent Ca2+ entry are complex. Thus, there is still some mileage in studying the effects of Ins(1,4,5)P3. Furthermore, mouse knock-out models of the β3 subunit of the voltage-gated Ca2+ channel (Berggren et al., 2004) or the IP3R adaptor protein, ankyrin B (Healy et al., 2010), suggest that there are still interesting aspects of the role of Ins(1,4,5)P3 in [Ca2+]i regulation to be illuminated. One drawback of all studies on Ins(1,4,5)P3, [Ca2+]i, and Ca2+-handling is that our information is based almost exclusively on cells and islets in vitro culture. Therefore, new in vivo systems have to be developed that can address this shortcoming. In this context, we have recently developed the anterior chamber of the eye as an imaging platform, which enables us to monitor longitudinally and noninvasively important events in islets at single cell resolution in the living organism (Speier et al., 2008a,b). An important future goal will be to carefully check the conclusions derived from experiments done on isolated islets and β-cells in vitro, especially as they relate to [Ca2+]i oscillations.

Another aspect of Ins(1,4,5)P3 to be examined is how the studies performed largely with rodent islets work with primary human islets. In this context, our recent work has indicated that many elements of the human islet anatomy and physiology are different from those of rodents (Cabrera et al., 2006; Jacques-Silva et al., 2010; Rodriguez-Diaz et al., 2011a,b). For example, although both human and rodent β-cells possess muscarinic receptors that are responsive to acetylcholine, the source of acetylcholine is strikingly different. In rodents, the source of this neurotransmitter is from parasympathetic nerve terminals, which are linked to vagal stimulation. In human islets, there is reduction of parasympathetic innervation, but the islet alpha cells have apparently taken this role, both synthesizing and releasing acetylcholine (Rodriguez-Diaz et al., 2011a,b). Although the muscarinic receptor’s downstream signaling is likely to be similar between rodents and humans, the temporal changes in concentration of acetylcholine may be different. As we have shown above, engagement of GPCRs and the liberation of Ins(1,4,5)P3 have important temporal dimensions, particularly when considering the [Ca2+]i oscillatory behavior that is fundamental to β-cell physiology and pathophysiology.

In thinking about the future of Ins(1,4,5)P3, it is important to also consider its receptor, some aspects of which are underexplored in the β-cell, particularly in relationship to its function as a primary location for multiple protein-protein interactions (Patterson et al., 2004). Of course, much can be extrapolated from the functioning of the same IP3Rs in other cell types, but when considering the different spectra of proteins present in different cell types, exploration of this aspect of IP3R function in the β-cell is lacking. As we have described earlier, when there is, for example, disruption of IP3R-linked anchoring proteins, such as ankyrin B (Healy et al., 2010), there is a considerable impact on IP3R function. This occurs in an impairment of the stimulus-secretion coupling of the β-cells in pancreatic islets, and aspects of a diabetic phenotype are generated. These studies underscore the need for further investigations of the IP3R in β-cells.

We have already discussed that InsP6, the ultimate downstream monoester polyphosphate of Ins(1,4,5)P3 metabolism, has important roles in the pancreatic β-cell, including exocytosis, endocytosis, and the regulation of the L-type voltage-dependent Ca2+ channel (see section III.B). However, two aspects have been little explored in this cell. These are the synthesis and breakdown of InsP6. We assume that the synthesis of InsP6 in β-cells is the same as in other mammalian cells, through the sequential phosphorylation of Ins(1,4,5)P3 by IPMK and Ins(1,3,4,5,6)P5 2-kinase (Fujii and Yok, 2005). The early embryonic lethality after the knockout of either of these two enzymes demonstrates the essential nature of InsP6 for life (Frederick et al., 2005; Verbisky et al., 2005).

We believe that IPMK, in particular, is likely to be an important, and as yet uncharacterized, player in β-cell regulation. This is because recent data from
other cell systems have highlighted IPMK as a multifunctional protein. In addition to its activity in the synthesis of Ins(1,3,4,5,6)P_{5}, IPMK can act as a PI3 kinase and convert PtdIns(4,5)P_{2} to PtdIns(3,4,5)P_{3} and, thus, activate both Akt/PKB (Maag et al., 2011) and presumably other PtdIns(3,4,5)P_{3}-regulated proteins. These new observations are of considerable interest in the regulation of β-cells, because Akt/PKB plays an essential role in both their physiology and their pathophysiology (Elghazi et al., 2006; Buzzi et al., 2010; Leibiger et al., 2010; Schultz et al., 2011). However, IMPK can operate noncatalytically through protein-protein interaction. Specifically, it regulates the mTor/Raptor complex (mTORC1) by increasing the strength of the association between mTor and raptor (Kim et al., 2011). Lack of IPMK compromises mTOR catalytic activity. Recent articles have indicated that an active mTORC1 complex is critical for β-cell proliferation (Balcazar et al., 2009). Thus, the study of IPMK in all its modes of operation (i.e., as an inositol phosphate and lipid kinase and as regulator of mTORC1), should be an important priority of future β-cell research. The Ins(1,3,4,5,6)P_{5} 2-kinase, presumably because of its production of InsP_{6}, has roles in the regulation of cilia (Sarmah et al., 2007) and mRNA processing (Brehm et al., 2007), both of which may be important in β-cell biology and should be explored. In keeping with IPMK, there appears to be a noncatalytic aspect to the function of Ins(1,3,4,5,6)P_{5} 2-kinase (Sarmah and Wente, 2009).

As is often the case, much less is known about the phosphatase(s) responsible for the dephosphorylation of InsP_{6}. multiple inositol polyphosphate phosphatase (MIPP), the only currently characterized phosphatase that degrades InsP_{6} in mammalian cells, is resident mostly in the ER (Ali et al., 1993; Craxton et al., 1997). Nonetheless, although it is apparently in a separate compartment from its principle substrates, evidence from the MIPP knockout mouse indicates that this enzyme can regulate Ins(1,3,4,5,6)P_{5} and InsP_{6} levels in vivo (Chi et al., 2000). More recent evidence from plants suggests that AtMRP5/AtABCC5, a member of the ABC multidrug resistance superfamily, specifically transports InsP_{6} into subcellular compartments (Nagy et al., 2009). Analogous proteins exist in mammalian cells; thus, there is now a potentially viable mechanism available by which MIPP may regulate cytosolic higher inositol phosphates. Intriguingly, MIPP, when expressed in the cytosol of pancreatic β-cells (Yu et al., 2003), can generate Ins(1,4,5)P_{3} from higher inositol phosphates, particularly Ins(1,3,4,5,6)P_{5}, a function it performs natively in organisms, such as Dictyostelium (Van et al., 1995). In the same experiments, Ins(1,2,3)P_{3} was generated from InsP_{6} (Yu et al., 2003). An important question to be verified is whether Ins(1,4,5)P_{3} or Ins(1,2,3)P_{3} can be produced in the lumen of the ER, where MIPP normally resides, and whether such a location has any relevant functional role. A physiologic role for Ins(1,4,5)P_{3} inside the ER is unlikely, because the binding site for Ins(1,4,5)P_{3} on the IP3R is exposed to the cytosol, not the lumen (Patterson et al., 2004; Mikoshiba, 2007). However, under conditions of stress, the ER structure can break down, for example, in palmitate models of the high-fat diet used to promote obesity-driven T2D (Borرادaile et al., 2006; Karaskov et al., 2006). Under these conditions, generation of Ins(1,4,5)P_{3} by a liberated MIPP could contribute to the apoptotic processes in which the IP3R is involved (Joseph and Hajnoczky, 2007). A physiologic role for the MIPP product Ins(1,2,3)P_{3} in the ER may be more likely, because its antioxidant properties may protect against ER stress. Finally, it is unlikely that MIPP is the only player in the metabolism of Ins(1,3,4,5,6)P_{5} and InsP_{6}; because of the vital nature of InsP_{6} metabolism documented here and in sections III., B and C, there is a limited phenotype in the MIPP knockout mouse (Chi et al., 2000). As expected, there is evidence from this knockout model of compensatory changes and the upregulation of alternative phosphatase activities. Therefore, it is likely that yet unidentified phosphatase(s) distinct from MIPP can also regulate Ins(1,3,4,5,6)P_{5} and InsP_{6} levels. One possible contender would be alkaline phosphatase, which, while being an ecto-enzyme, spends some of its life in the ER on route to the plasma membranes. In support of this idea, studies on osteogenesis indicate that there is some reciprocal expression between the two enzymes (Hidaka et al., 2003). Another possibility is PTEN, the phosphatidylinositol 3,4,5-trisphosphate phosphatase (Leslie et al., 2012). However, this enzyme can degrade Ins(1,3,4,5,6)P_{5}, but not InsP_{6} (Caffrey et al., 2001). Furthermore, its ability to regulate Ins(1,3,4,5,6)P_{5} levels in intact cells is controversial (Orchiston et al., 2004; Deleu et al., 2006).

In terms of higher inositol phosphates, the most interesting current arena is the inositol pyrophosphates. The clear link between PP-InsP_{5} or IP_{7}, and the β-cell exocytotic potential is an exciting finding (Illies et al., 2007). As we have hypothesized earlier in this review, this finding, together with other published information on the enzymatic properties of IP6Ks, suggests that IP6K1 is a potentially important metabolic sensor and, thus, a novel link between T2D and the β-cell secretory defect. An important remaining question is whether IP6K1 functions to couple energy metabolism, specifically glycolysis, to secretion and, in that case, how that is achieved. Two important pieces of information are lacking: one related to topography and the second related to mechanism. With regard to the topography, the insights from the knockdown of IP6K1 (Illies et al., 2007) suggest that this enzyme has a unique role that we believe must relate to its particular cellular location. This site is apparently distinct from the location of IP6K2. In β-cell expression of a GFP-tagged IP6K1 does not reveal a particular
location of IP6K1 (G. C. Gaboardi, P.-O. Berggren, and C. J. Barker, unpublished observations). However, it is possible that the presence of the GFP tag may sterically hinder protein–protein interactions that can occur endogenously. It is equally likely that the exogenous overexpressed protein floods the cell, and thus, it may be difficult to observe the compartmentalization apparently expressed by the endogenous protein. Furthermore, we know that the overexpression of any of the three IP6Ks is equally effective in affecting the RRP (Illies et al., 2007); thus, under such expression conditions, the unique position of IP6K1 is likely to be masked.

An article published during the final stages of preparation of this review further highlights the connection between energy metabolism and inositol pyrophosphates and underscores the idea that glycolysis is intimately linked to inositol pyrophosphates. The study (Szijgyarto et al., 2011) mostly relates to yeast, but some confirmatory experiments have been done in mouse embryonic fibroblasts derived from IP6K1 knockout mice. There are, however, no data from more specialized mammalian cells. The striking observation made is that knockout of the only yeast IP6K, kcs1, results in mitochondrial dysfunction with a paradoxical elevation of cellular ATP levels to threefold their normal levels. This increased ATP was found to be attributable to an enhanced glycolysis. The upregulation of glycolytic ATP production and the associated relegation of mitochondrial ATP generation is called the Warburg effect (Warburg, 1956) and is a well-known phenomenon. In this new study on inositol pyrophosphates, a molecular mechanism was proposed for the increased ATP production by glycolysis after IP6K knockdown. In yeast, the transcription of the genes that control glycolysis is under the coordinated regulation of a common transcriptional element. An element in this transcriptional unit, a transcription factor called GCR1, is pyrophosphorylated by inositol pyrophosphates that lead to a repression of transcription. When levels of pyrophosphates are depleted after knockout of the IP6K, pyrophosphorylation is lost, and this enables the association of the activating protein, GCR2, with the transcriptional unit and the upregulation of glycolysis. One of several possible interpretations is that the upregulation of glycolysis is also an attempt by the cell to adapt to the loss of inositol pyrophosphate production. This scenario suggests that glycolytic ATP may be important in the generation of inositol pyrophosphates. In other words, the yeast futilely upregulates the pathway that produces pyrophosphates to restore function. Whatever the explanation, this new study highlights that the relationship between pyrophosphates and glycolysis is an important one to pursue in pancreatic β-cells and other cells in which glucose metabolism has an important regulatory role.

Mechanism of action is still an important unknown with regard to β-cell PP-InsP₅. As we have discussed earlier, this is compounded by the fact that at least two modes of operation are exhibited by pyrophosphates, namely pyrophosphorylation and allosteric regulation. The 5-PP-InsP₅ that is generated by the IP6Ks is associated with only one current known form of allosteric regulation, and that is the negative regulation of Akt/PKB (Chakraborty et al., 2010). Unfortunately, mechanistically, the only role associated with PKB in the exocytotic process is a positive one (Leibiger et al., 2010); thus, removal of PP-InsP₅ should enhance, not compromise, exocytosis if PKB is involved. Because this is not the case, it is hard to see PKB as a link between PP-InsP₅ and insulin secretion. More uncertainty surrounds pyrophosphorylation, which is still fraught by some degree of controversy (Shears et al., 2011). There is a lack of specificity in the pyrophosphorylation process (i.e., different inositol pyrophosphate species seem to be equally effective) (Bhandari et al., 2007). Therefore, when a pyrophosphate-regulated event shows little selectivity between different isoforms, this could be indicative of a pyrophosphorylation mode of action. The regulation of insulin exocytosis by 5-PP-InsP₅ (Illies et al., 2007) is such a case, because several of the PP-InsP₅ isoforms tested were also effective in promoting exocytosis. However, none of the currently identified pyrophosphorylated proteins are positively associated with either exocytosis or vesicle trafficking, although the β3A or B form of the AP3 adaptor protein complex when pyrophosphorylated has a negative effect on vesicle trafficking (Azevedo et al., 2009).

One other possible explanation for the lack of specificity of the PP-InsP₅ isoforms is that the effect is mediated by another pyrophosphate altogether. This would have to be a metabolite of the original 5-PP-InsP₅, and thus, the most likely candidate would be the so called IP8, or 1,5-bis-diphosphoinositol tetrakisphosphate 1,5-(PP)₂-InsP₄ (Barker et al., 2009b; Shears, 2009; Saiardi, 2012). This molecule is attractive, because it possesses some of the same stereochemistry as multiple PP-InsP₅ isomers. As we have mentioned earlier, the only material with the potential elution properties of an IP₅ that can be detected in β-cells is at very low concentrations, compared with 5-PP-InsP₅ (C. J. Barker, C. Illies, and P.-O. Berggren, unpublished results). Therefore, to be a replacement for PP-InsP₅, it would need to be very potent.

Is there a possibility of even more pyrophosphorylated inositol polyphosphates, an IP₉ or an IP₁₀, for example? In vitro IP6Ks can produce multiple highly phosphorylated inositol derivatives beyond IP₈ (Draskovic et al., 2008; Azevedo et al., 2010), and certainly, overexpression of IP6K1 in some β-cell lines can...
generate very polar materials, consistent with such inositol pyrophosphates (Barker et al., 2009b) (C. Illies, P.-O. Berggren, and C. J. Barker, unpublished data). However, we do not see these materials in extractions of untransfected cells. A known property of IP6Ks is to sequentially add additional phosphate groups to an already pyrophosphorylated position, forming phosphate strings (Draskovic et al., 2008; Azevedo et al., 2010). These kinds of molecules are, however, far removed structurally from the diphosphoinositol inositol structures that comprise the classic inositol pyrophosphates. The reason that these inositol phosphate string molecules are not seen under normal physiologic conditions is probably the presence of the very active diphosphoinositol polyphosphate phosphohydrolases (DIPPs) in cells (Safrany et al., 1998; Barker et al., 2009b; Shears, 2009). This raises the idea that DIPP could be a key regulator of pyrophosphate metabolism in pancreatic β-cells and, thus, represents the potential final frontier in inositol pyrophosphate research and a fitting place to close this review.

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Inositol Polyphosphates in Pancreatic β-Cells


