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## ***Oscillatory control of insulin secretion***

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 $\text{PI3-kinase}$ ,  $\text{PIP}_3$

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## **Abstract**

Pancreatic  $\beta$ -cells possess an inherent ability to generate oscillatory signals that trigger insulin release. Coordination of the secretory activity among  $\beta$ -cells results in pulsatile insulin secretion from the pancreas, which is considered important for the action of the hormone in the target tissues. This review focuses on the mechanisms underlying oscillatory control of insulin secretion at the level of the individual  $\beta$ -cell. Recent studies have demonstrated that oscillations of the cytoplasmic  $\text{Ca}^{2+}$  concentration are synchronized with oscillations in  $\beta$ -cell metabolism, intracellular cAMP concentration, phospholipase C activity and plasma membrane phosphoinositide lipid concentrations. There are complex interdependencies between the different messengers and signalling pathways that contribute to amplitude regulation and shaping of the insulin secretory response to nutrient stimuli and neurohormonal modulators. Several of these pathways may be important pharmacological targets for improving pulsatile insulin secretion in type 2 diabetes.

### **1. Pulsatile insulin secretion**

Since insulin is the only blood glucose-lowering hormone, its secretion is essential for glucose homeostasis, and disturbances are associated with glucose intolerance and diabetes. Glucose is the major stimulus for insulin release but secretion is enhanced also by other nutrients and is under stimulatory and inhibitory control by hormones and neurotransmitters. Although the external factors are essential determinants of insulin secretion, there are also input-independent variations in hormone release. Regular oscillations of the circulating insulin concentrations in normal subjects consequently occur without accompanying changes

in plasma glucose and reflect a pacemaker function of the pancreas (Lang et al., 1981; Matthews et al., 1983a). Studies in normal subjects with suppressed endogenous insulin secretion and diabetic patients have indicated that less insulin is required to maintain normoglycaemia if the hormone is infused in an oscillatory manner compared to a constant rate (Matthews et al., 1983b; Bratusch-Marrain et al., 1986; Matthews et al., 1987; Paolisso et al., 1988a; Paolisso et al., 1988b). The better efficiency of oscillatory insulin delivery is probably due to higher expression of insulin receptors (Goodner et al., 1988). Interestingly, this characteristic insulin pattern is early deteriorated in patients with type 2 diabetes (Lang et al., 1981; Matthews, 1987) and their close relatives (O'Rahilly et al., 1988). Therefore, loss of insulin oscillations may contribute to insulin resistance that needs to be compensated by hypersecretion of the hormone. The increased insulin demand may exhaust the pancreatic  $\beta$ -cells and cause overt type 2 diabetes in susceptible individuals.

The insulin oscillations are generated by pulsatile secretion from the pancreas. The oscillations are most prominent in the portal vein, which delivers blood from the pancreas to the liver. Measurements in the portal vein of dogs and humans have indicated that virtually all insulin (>70-75%) is released in a pulsatile fashion (Pørksen et al., 1995; Pørksen et al., 1997). Early estimates of pulse intervals are somewhat variable due to technical limitations but later data indicate a periodicity of 4-6 min in humans (Storch et al., 1993; Pørksen et al., 1997; Ritzel et al., 2001) and dogs (Pørksen et al., 1996). The insulin-releasing  $\beta$ -cells are located in about 1 million islets of Langerhans distributed within the 25 cm long human pancreas. It is evident that pulsatile secretion requires a formidable coordination of the secretory activity among islets and  $\beta$ -cells. Since this coordination occurs in the absence of external neuronal input to the pancreas but can be temporarily disrupted by neurotransmitters,

it was early suggested to involve coordination by intrapancreatic neurons (Stagner et al., 1980). Later experiments confirmed that pulsatile secretion is prevented by neural blockade (Stagner and Samols, 1985a; Gylfe et al., 2000). However, the neurons involved do not seem to be adrenergic or cholinergic (Stagner and Samols, 1985b). Studies of the synchronization of  $\text{Ca}^{2+}$  signalling among dispersed  $\beta$ -cells by humoral factors have led to proposals that ATP (Grapengiesser et al., 1999; Grapengiesser et al., 2004; Hellman et al., 2004; Grapengiesser et al., 2005) and/or the gases NO (Grapengiesser et al., 1999; Grapengiesser et al., 2001) and CO (Lundquist et al., 2003) released from neurons act to coordinate pulsatile insulin secretion (see below).

Insulin secretion remains pulsatile in isolated individual pancreatic islets from mice (Bergsten and Hellman, 1993b; Bergsten and Hellman, 1993a; Gilon et al., 1993; Bergsten et al., 1994; Bergsten, 1995; Gilon and Henquin, 1995) and humans (Hellman et al., 1994) with a dominating frequency similar to that of secretion from the pancreas. The rhythmic variations in secretion from islets are synchronized with oscillations of the cytoplasmic  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ; (Gilon et al., 1993; Bergsten et al., 1994; Bergsten, 1995; Gilon and Henquin, 1995)), which is the most important trigger of insulin release (see below). Studying  $[\text{Ca}^{2+}]_i$  signalling in clusters of mouse  $\beta$ -cells exposed to intermediate stimulating concentrations of glucose some adjacent cells were oscillating in synchrony whereas others were silent (Gylfe et al., 1991). Further increase of the glucose concentration resulted in spreading of the synchronized oscillations to previously silent cells, and it was possible to follow signal propagation between the cells. This experiment illustrates the importance of  $\beta$ -cell recruitment and intercellular coupling for glucose regulation of the insulin pulse amplitudes. It has been known for a long time that  $\beta$ -cells are electrically coupled by gap

junctions, and the dominating intercellular channel was later identified as connexin 36 (Ravier et al., 2005). Opinions differ about the size of electrically coupled  $\beta$ -cell clusters within pancreatic islets ranging from relatively few cells to a functional syncytium involving all  $\beta$ -cells (Bavamian et al., 2007). These differences may perhaps be explained by variations in coupling at different functional states. However, spreading of the depolarization underlying rise of  $[Ca^{2+}]_i$  (see below) by gap junctional coupling is certainly the most important explanation for synchronization of pulsatile insulin release between  $\beta$ -cells in the islets of Langerhans.

The kinetics of insulin secretion can be studied with relatively good time resolution from individual pancreatic islets using traditional immunoassays. Unfortunately, such techniques are not sufficiently sensitive to characterize the kinetics of secretion from individual  $\beta$ -cells. Patch clamp recordings of changes in plasma membrane capacitance with fusion of insulin secretory granules have sufficient sensitivity but are complicated by endocytosis events and are best for determining the effects of imposed changes in membrane potential (Ämmälä et al., 1993c). Amperometric detection of serotonin released together with insulin from preloaded  $\beta$ -cells can be used to detect secretion events close to the fibre (Kennedy et al., 1993) but does not provide information about total secretion. Using an extracellular indicator that detects  $Zn^{2+}$  co-secreted with insulin, it was possible to study pulsatile insulin release in parallel with synchronized oscillations of  $[Ca^{2+}]_i$  in small clusters of  $\beta$ -cells (Qian et al., 2004). Pulsatile insulin secretion synchronous with oscillations in  $[Ca^{2+}]_i$  has also been detected in individual human  $\beta$ -cells with total internal reflection (TIRF) microscopy of fluorescent cargo protein expressed in the secretory granules (Michael et al., 2007). An alternative TIRF-based approach detecting formation of phosphatidylinositol 3,4,5-

trisphosphate (PIP<sub>3</sub>) in response to autocrine activation of insulin receptors (Idevall-Hagren and Tengholm, 2006) allowed detection of synchronous oscillations of [Ca<sup>2+</sup>]<sub>i</sub> and pulsatile insulin release from individual clonal β-cells with similar frequency as pulsatile secretion *in vivo*. Figure 1 shows insulin oscillations at different organizational levels. Since major features of pulsatile insulin secretion from single β-cells *in vitro* are similar to secretion from the pancreas *in vivo*, we will evade some of the complexity and concentrate the subsequent discussion on factors determining pulsatile secretion in the individual β-cell.

## 2. Role of metabolism for glucose-induced insulin secretion

It was early suggested that glucose is recognized as an insulin secretagogue by being metabolized by the β-cells (Grotsky et al., 1963; Coore and Randle, 1964). This idea, referred to as “the substrate site hypothesis” (Randle et al., 1968), slowly became widely accepted. An early key observation was that glucose-stimulated insulin secretion is prevented by mannoheptulose, a competitive inhibitor of glucose phosphorylation (Coore and Randle, 1964). It was not until 20 years later after the invention of the patch clamp technique that ATP (Ashcroft et al., 1984; Cook and Hales, 1984) or the ATP/ADP ratio (Ashcroft and Rorsman, 1989) could be identified as a primary factor coupling glucose metabolism to insulin secretion. ATP acts by inhibiting ATP-sensitive K<sup>+</sup> (K<sub>ATP</sub>) channels, thus confirming the early and innovative proposal that glucose depolarizes the β-cells by inhibiting the K<sup>+</sup> permeability (Sehlin and Täljedal, 1975). It is this depolarization that raises [Ca<sup>2+</sup>]<sub>i</sub> and triggers secretion by opening voltage-dependent Ca<sup>2+</sup> channels (see below). However, there may be additional mechanisms contributing to depolarization, since glucose can still stimulate some voltage-dependent Ca<sup>2+</sup> entry in β-cells lacking functional K<sub>ATP</sub> channels (Szollosi et al., 2007).



The dose-response relationship for glucose-induced insulin secretion is sigmoidal with a species-dependent stimulatory threshold at 3-7 mM and a steep increase, reaching maximum at 15-30 mM of the sugar (Henquin et al., 2006; Salehi et al., 2006). Such glucose dependence is consistent with the fact that high  $K_m$  GLUT2 is the dominating glucose transporter in rodent  $\beta$ -cells (Chen et al., 1990; Johnson et al., 1990). It was therefore unexpected that human  $\beta$ -cells preferentially express low  $K_m$  GLUT1 (De Vos et al., 1995), whose capacity is close to saturation already at threshold concentrations of glucose. However, consistent with the latter observation, transgenic re-expression of GLUT1 or GLUT2 into  $\beta$ -cells of GLUT2-null mice were equally efficient in restoring normal glucose sensing (Thorens et al., 2000). The explanation is probably a very high glucose transport capacity that is not rate limiting. Indeed, the uptake is so rapid that reduction of the temperature to 8 °C was required in the first demonstration of a carrier-mediated saturable uptake of glucose into pancreatic islets (Hellman et al., 1971). The rate-limiting glucosensor that determines the characteristic sigmoidal glucose concentration dependence of insulin secretion is instead the first phosphorylation step in glucose metabolism exerted by the high  $K_m$  glucokinase (Matschinsky, 1990).

Early studies emphasized glycolysis as a likely origin of factors coupling glucose metabolism to insulin secretion (Hellman, 1970). However, the discovery that  $\beta$ -cell depolarization with increase of  $[Ca^{2+}]_i$  depends on ATP (Ashcroft et al., 1984; Cook and Hales, 1984) shifts attention towards oxidative metabolism as most ATP is produced by mitochondria. Since secretion is pulsatile it is obvious that oscillations in metabolism and ATP generation may underlie this pattern. Indeed, there is ample evidence supporting the occurrence of metabolic oscillations. Parallel oscillations in oxygen consumption and  $[Ca^{2+}]_i$

were first found in studies of groups of perfused islets (Longo et al., 1991). Oxygen oscillations with similar temporal properties as those of  $[Ca^{2+}]_i$  have later been demonstrated with intra-islet electrodes (Jung et al., 1999a; Jung et al., 1999b; Jung et al., 2000; Ortsäter et al., 2000), and found to correlate with oscillations in glucose consumption (Jung et al., 2000) and insulin release (Ortsäter et al., 2000). Oscillations in NAD(P)H have been observed in single rat  $\beta$ -cells (Pralong et al., 1994) but have been difficult to catch in islets (Panten et al., 1973; Gilon and Henquin, 1992). It was not until recently that NAD(P)H oscillations were convincingly demonstrated in islets (Luciani et al., 2006) where they are coupled to the  $[Ca^{2+}]_i$  oscillations. Also the mitochondrial membrane potential oscillates in parallel with  $[Ca^{2+}]_i$  (Krippeit-Drews et al., 2000) and with temporal characteristics similar to that of the NAD(P)H oscillations (Luciani et al., 2006). Direct observations of oscillations in ATP have been made both in individual mouse and human  $\beta$ -cells (Ainscow and Rutter, 2002), and there is evidence for oscillations in the activity of the  $K_{ATP}$  channels (Dryselius et al., 1994; Larsson et al., 1996), which are probably the most important ATP targets in stimulus-secretion coupling.

There are different ideas how oscillations in metabolism are generated. According to one hypothesis oscillations are an inherent property of metabolism (Tornheim, 1997). Another possibility involves feedback effects of  $Ca^{2+}$  on metabolism (Detimary et al., 1998; Magnus and Keizer, 1998a; Magnus and Keizer, 1998b; Jung et al., 2000; Krippeit-Drews et al., 2000; Kindmark et al., 2001; Ainscow and Rutter, 2002; Luciani et al., 2006; Bertram et al., 2007a). According to the former alternative the metabolic oscillations originate in glycolysis due to allosteric feedback activation of the “oscillatory” isoform of phosphofructokinase (PFK-M) in  $\beta$ -cells (Tornheim, 1997). However, oscillations in  $[Ca^{2+}]_i$  remained normal after 95-98%

suppression of PFK-M activity in mouse islets (Richard et al., 2007). Moreover, oscillations of  $[Ca^{2+}]_i$  are not only generated by glucose, but also by mitochondrial substrates like leucine (Grapengiesser et al., 1989b),  $\alpha$ -ketoisocaproic acid (Martin et al., 1995; Heart and Smith, 2007) and methyl pyruvate (Heart and Smith, 2007) even in the absence of glycolytic flux (Heart and Smith, 2007), although some of these observations are controversial (Lenzen et al., 2000; Dahlgren et al., 2005). Since citrate oscillates in isolated islet mitochondria, it has also been proposed that there is an independent mitochondrial oscillator and that exported citrate may feedback-coordinate mitochondrial and glycolytic oscillations (MacDonald et al., 2003).

As increased metabolism depolarizes the  $\beta$ -cells and raises  $[Ca^{2+}]_i$  it seems likely that primary oscillations in metabolism drives those of  $[Ca^{2+}]_i$ . However, there are also mechanisms by which  $Ca^{2+}$  may feedback-regulate metabolism. Rise of  $[Ca^{2+}]_i$  thus increases ATP consumption due to energy-demanding elimination of the cation from the cytoplasm (Detimary et al., 1998). In addition,  $Ca^{2+}$  decreases ATP production by depolarizing the mitochondrial inner membrane by its uptake via the uniporter (Magnus and Keizer, 1997; Magnus and Keizer, 1998b; Krippeit-Drews et al., 2000). The situation is further complicated by the fact that  $Ca^{2+}$  stimulates metabolism by activating mitochondrial dehydrogenases (McCormack et al., 1990; Civelek et al., 1996; Pitter et al., 2002) and that  $Ca^{2+}$  may regulate glycolytic enzymes (Jung et al., 2000). Metabolic oscillations may indeed reflect intrinsic oscillatory mechanisms in glucose metabolism, but the oscillations are modified both by stimulatory and inhibitory feedback effects of  $Ca^{2+}$  (Luciani et al., 2006; Bertram et al., 2007b).

So far the discussion about metabolism has been focussed on the classical  $K_{ATP}$  channel-dependent pathway leading to depolarization, rise of  $[Ca^{2+}]_i$  and triggering of insulin release.

However, some of the concentration-dependent stimulatory effect of glucose on insulin secretion remains when  $[Ca^{2+}]_i$  is elevated under conditions when the  $K_{ATP}$  channels are either blocked or held open (Henquin, 2000) or after knocking out functional  $K_{ATP}$  channels (Nenquin et al., 2004; Szollosi et al., 2007). This  $K_{ATP}$  channel-independent process is mostly considered as an amplification mechanism, which remains functionally silent unless  $[Ca^{2+}]_i$  is elevated. Yet other studies have provided evidence that the  $K_{ATP}$  channel-independent pathway may also exhibit triggering properties, since glucose under some conditions stimulates insulin release in a  $Ca^{2+}$ -independent manner (Komatsu et al., 1995; Komatsu et al., 1996; Komatsu et al., 2001). The  $K_{ATP}$  channel-independent pathway has remained elusive and many mechanisms have been proposed (reviewed in (Komatsu et al., 2001)). Changes in ATP concentration is an attractive explanation, since the nucleotide not only closes the  $K_{ATP}$  channels, but also provides energy for a large number of processes, like insulin granule transport to the plasma membrane (Varadi et al., 2002; Varadi et al., 2003; Varadi et al., 2005) and priming of the granules prior to exocytosis (Eliasson et al., 1997). The ATP-derived product cAMP is another appealing alternative, since it is probably the most important stimulatory messenger after  $Ca^{2+}$  (see below). The observations that pulsatile insulin release is maintained under basal conditions with no elevation of  $[Ca^{2+}]_i$  (Westerlund et al., 1996) or when  $[Ca^{2+}]_i$  is elevated to a stable level (Westerlund et al., 1997) indicate that metabolic oscillations do not necessarily require  $Ca^{2+}$  feedback and that such oscillations promote pulsatile secretion via the  $K_{ATP}$  channel-independent pathway.

### 3. The cytoplasmic $\text{Ca}^{2+}$ concentration

It has been known for more than 40 years that extracellular  $\text{Ca}^{2+}$  is a prerequisite for glucose-stimulated insulin secretion (Grodsky and Bennett, 1966; Milner and Hales, 1967) and numerous later studies have emphasized the fundamental role of  $\text{Ca}^{2+}$  in the insulin secretory process (Wollheim and Sharp, 1981; Hellman and Gylfe, 1986b; Ashcroft and Rorsman, 1989).  $\text{Ca}^{2+}$  stimulates insulin secretion by regulating docking and initiating fusion of secretory granules with the plasma membrane, a process mediated by SNARE proteins (Rorsman and Renström, 2003; MacDonald and Rorsman, 2007). Voltage-dependent  $\text{Ca}^{2+}$  entry is actually directed to the sites of exocytosis via the binding of the L-type  $\text{Ca}^{2+}$  channels to SNARE proteins (Wiser et al., 1999). Release of  $\text{Ca}^{2+}$  from intracellular stores, on the other hand, is essential for the amplification of insulin secretion by promoting the replenishment of the readily releasable pool of secretory granules (Gromada et al., 1999).

In 1988 it was discovered that glucose-stimulated individual  $\beta$ -cells show pronounced  $[\text{Ca}^{2+}]_i$  oscillations with a periodicity of 2-6 min (Grapengiesser et al., 1988b). These slow  $[\text{Ca}^{2+}]_i$  oscillations are classical membrane oscillations that depend on the presence of extracellular  $\text{Ca}^{2+}$  and are inhibited by blockers of voltage-dependent  $\text{Ca}^{2+}$  channels ((Grapengiesser et al., 1989a); Table 1). As discussed above the depolarization is due to increased ATP/ADP ratio and closure of  $\text{K}_{\text{ATP}}$  channels. The electrophysiological events resulting in slow  $[\text{Ca}^{2+}]_i$  oscillations in individual  $\beta$ -cells have been difficult to demonstrate. So far there is only one study reporting that each slow  $[\text{Ca}^{2+}]_i$  oscillation is paralleled by an extended burst of electrical activity with action currents ((Dryselius et al., 1999); Figure 2C). The initial rise of  $[\text{Ca}^{2+}]_i$  is preceded by a lowering ((Grapengiesser et al., 1988a); Figure 2A) that essentially reflects ATP-stimulated  $\text{Ca}^{2+}$  sequestration in the endoplasmic reticulum (ER;

(Tengholm et al., 1999)) that becomes masked when depolarization has reached the threshold for voltage-dependent  $\text{Ca}^{2+}$  entry (Chow et al., 1995). The lowering is prevented by inhibiting the sarco(endo)plasmic reticulum  $\text{Ca}^{2+}$  ATPase (SERCA; (Chow et al., 1995)) and has been attributed to a high affinity SERCA2 mechanism, since it remains after ablation of the low affinity SERCA3 transporter (Arredouani et al., 2002).

Although intracellular sequestration and release of  $\text{Ca}^{2+}$  from the ER does not seem to be an absolute requirement for generating slow  $[\text{Ca}^{2+}]_i$  oscillations (Liu et al., 1996), these processes contribute to shaping the oscillations (Fridlyand et al., 2003). SERCA inhibition (Gilon et al., 1999) or selective SERCA3 ablation (Arredouani et al., 2002) consequently accelerates both the rising and declining phases of each oscillation and increases the amplitude, indicating that the ER acts as a low affinity passive  $\text{Ca}^{2+}$  buffer to smooth the  $[\text{Ca}^{2+}]_i$  oscillations. There is also gated release of  $\text{Ca}^{2+}$  from the ER via inositol 1,4,5-trisphosphate receptors ( $\text{IP}_3\text{Rs}$ ) causing pronounced  $[\text{Ca}^{2+}]_i$  spikes (Table 1). Such release can be triggered after activation of phosphoinositide-specific phospholipase C (PLC) by G-protein coupled hormone receptors (Grapengiesser et al., 1989a). Also elevation of cAMP promotes irregular  $\text{Ca}^{2+}$  spiking that is superimposed on top of the slow oscillations or on a stably elevated  $[\text{Ca}^{2+}]_i$  level (Grapengiesser et al., 1991; Liu et al., 1996; Dyachok and Gylfe, 2004), reflecting facilitation of  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (CICR) via  $\text{IP}_3\text{Rs}$  (Dyachok and Gylfe, 2004).  $\text{Ca}^{2+}$  spiking is sufficiently pronounced to activate a hyperpolarizing  $\text{K}^+$  current that can arrest action potential firing (Ämmälä et al., 1993b; Dryselius et al., 1999) and provide a negative feedback on the slow  $[\text{Ca}^{2+}]_i$  oscillations (Bertram et al., 2007a; Bertram et al., 2007b) independent of the  $\text{Ca}^{2+}$  effects on metabolism discussed above (Figure 2D).

Interestingly,  $\text{Ca}^{2+}$  spikes resulting from intracellular  $\text{Ca}^{2+}$  mobilization can be synchronized among isolated cells lacking physical contact (Grapengiesser et al., 1999) Figure 2B). These oscillations are sensitive to purinergic P2 receptor antagonists, indicating that  $\beta$ -cells communicate via release of ATP (Hellman et al., 2004). Since a distinct  $[\text{Ca}^{2+}]_i$  spike results in exocytotic release of ATP co-stored with insulin (Dyachok and Gylfe, 2004; Braun et al., 2007), intercellular diffusion of the nucleotide can result in propagation of  $[\text{Ca}^{2+}]_i$  spikes between cells lacking physical contact (Hellman et al., 2004). The implication of this intercellular communication for oscillatory control of insulin secretion is that the  $[\text{Ca}^{2+}]_i$  spikes can synchronize the slow glucose-induced  $\text{Ca}^{2+}$  oscillations by resetting their phase relationship (Grapengiesser et al., 2004). This mechanism may serve as a complement to synchronization by gap junctional coupling within the islet, but is probably more important for the entrainment of different islets to a common rhythm. The synchronizing spiking among islets can be initiated by ATP released from intrapancreatic neurons (Salehi et al., 2005).

Isolated pancreatic islets not only show slow oscillations of  $[\text{Ca}^{2+}]_i$  but often several-fold faster regular oscillations or slow oscillations with superimposed fast ones ((Bergsten et al., 1994; Liu et al., 1998); Table 1). Also the fast oscillations correlate with cyclic membrane depolarization with bursts of electrical activity (Santos et al., 1991). They somehow co-depend on the  $\text{Ca}^{2+}$  handling by the ER, since fast islet oscillations are immediately transformed into slow ones by SERCA inhibition (Liu et al., 1998). Like the slow  $[\text{Ca}^{2+}]_i$  oscillations, the fast ones generate pulsatile insulin release from individual pancreatic islets (Bergsten, 1995). However, pulsatile insulin release from the pancreas does not show this fast component probably because the fast bursting is not synchronized between islets in the

pancreas (Valdeolmillos et al., 1996). Therefore, the complex mechanisms underlying the fast islet oscillations will not be further discussed.

#### **4. PIP<sub>2</sub> and signalling via phospholipase C**

Phosphatidylinositol-4,5-bisphosphate (PIP<sub>2</sub>) constitutes only ~1% of the phospholipids in the inner leaflet of the plasma membrane (McLaughlin et al., 2002), but nevertheless plays an important role in secretion by regulating vesicle priming and trafficking (Olsen et al., 2003; Gromada et al., 2005; Waselle et al., 2005) and by serving as precursor for second messengers. One of the best known functions of PIP<sub>2</sub> is as substrate for PLC generation of IP<sub>3</sub> and diacylglycerol (DAG; (Berridge et al., 2000)). Whereas IP<sub>3</sub> mobilizes Ca<sup>2+</sup> from the ER, DAG plays an important role as activator of protein kinase C (PKC). PLC activity was early detected in rodent islets (Schrey and Montague, 1983; Dunlop and Larkins, 1986), and subsequent analyses have demonstrated islet expression of several PLC- $\beta$ , - $\gamma$ , and - $\delta$  isozymes (Kelley et al., 1995; Zawalich et al., 1995; Gasa et al., 1999; Zawalich and Zawalich, 2000; Kim et al., 2001a; Kim et al., 2001b). The importance of PLC activity for insulin secretion is underlined by the fact that the enzyme is activated not only after exposure of islets and  $\beta$ -cells to various G-protein coupled receptor stimuli, such as acetylcholine/carbachol (Best and Malaisse, 1983; Hellman and Gylfe, 1986a; Best et al., 1987; Biden et al., 1987; Gilon and Henquin, 2001) and ATP (Gylfe and Hellman, 1987; Blachier and Malaisse, 1988), but also after exposure to glucose (Axen et al., 1983; Best and Malaisse, 1983; Laychock, 1983; Montague et al., 1985) and depolarizing agents (Laychock, 1983; Mathias et al., 1985; Best et al., 1987; Biden et al., 1987; Zawalich and Zawalich, 1988).



$\beta$ -cells often respond to PLC-activating receptor agonists with oscillations of  $[Ca^{2+}]_i$  (Prentki et al., 1988; Gylfe, 1991; Hellman et al., 1992; Theler et al., 1992; Miura et al., 1996) and these oscillations are characterized by a much shorter period than the glucose-induced, slow oscillations described above. Most of the  $[Ca^{2+}]_i$ -elevating effect is due to  $IP_3$ -mediated  $Ca^{2+}$  mobilization from the ER, but the emptying of the stores also triggers  $Ca^{2+}$  entry through store-operated channels in the plasma membrane (Liu and Gylfe, 1997; Miura et al., 1997; Dyachok and Gylfe, 2001). Although the store-operated influx of  $Ca^{2+}$  only causes a small elevation of  $[Ca^{2+}]_i$ , this pathway may also contribute by its depolarizing effect. In this regard, it is pertinent that  $Na^+$  can permeate the store-operated channels (Worley et al., 1994a; Worley et al., 1994b). Increased  $Na^+$  entry actually explains the depolarizing effect of muscarinic stimulation that triggers voltage-dependent  $Ca^{2+}$  entry (Gagerman et al., 1980; Henquin et al., 1988; Saha and Hellman, 1991; Gilon and Henquin, 1993; Gilon and Henquin, 2001), although  $Na^+$  channels other than store-operated channels probably contribute to this depolarization (Miura et al., 1996; Gilon and Henquin, 2001).

Over the last decade it has become possible to image the kinetics of PLC activity in individual cells using  $PIP_2$ - and/or  $IP_3$ -binding protein domains fused to the green fluorescent protein or its colour variants (Varnai and Balla, 2006). Such reporter proteins shuttle between the plasma membrane and cytoplasm depending on the relative concentrations of  $PIP_2$  and  $IP_3$  in the two compartments, and this translocation serves as an indirect read-out for PLC activity (Figure 3A). Kinetic analysis of PLC activity in single  $\beta$ -cells has revealed that receptor-induced PLC activity is under tight dynamic control of  $[Ca^{2+}]_i$  (Thore et al., 2005). In mouse insulinoma cells, carbachol was found to activate PLC in two distinct phases, with a rapid and transient first phase that was amplified several-fold by  $Ca^{2+}$  released from intracellular stores,

followed by a second phase sustained by store-operated  $\text{Ca}^{2+}$  entry ((Thore et al., 2005); Figure 3B). The same study demonstrated that feedback activation of PLC by  $\text{Ca}^{2+}$  mobilized from intracellular stores is part of the mechanism by which cAMP generates  $[\text{Ca}^{2+}]_i$  spikes in mouse  $\beta$ -cells. The importance of such spikes for membrane repolarization and synchronization of  $\beta$ -cell oscillatory activity is discussed above.

The activation of islet PLC by glucose (Axen et al., 1983; Best and Malaisse, 1983; Laychock, 1983; Montague et al., 1985) and depolarizing agents (Laychock, 1983; Mathias et al., 1985; Best et al., 1987; Biden et al., 1987; Zawalich and Zawalich, 1988) is mainly due to depolarization and  $\text{Ca}^{2+}$  influx, although some glucose-stimulated  $\text{PIP}_2$  hydrolysis was reported to proceed even under conditions when elevation of  $[\text{Ca}^{2+}]_i$  was prevented (Best and Malaisse, 1983; Kelley et al., 1995). Real-time imaging studies in isolated insulinoma cells and intact islets (Thore et al., 2004; Tamarina et al., 2005; Thore et al., 2007) have recently confirmed that glucose stimulates PLC activity secondary to the elevation of  $[\text{Ca}^{2+}]_i$  and that membrane depolarization alone (Gromada et al., 1996; Liu et al., 1996) or increase of  $[\text{Ca}^{2+}]_i$  (Mathias et al., 1985; Best et al., 1987; Biden et al., 1987) are sufficient to activate the enzyme. Depolarization with accompanying elevation of  $[\text{Ca}^{2+}]_i$  is particularly effective in activating PLC, and  $[\text{Ca}^{2+}]_i$  oscillations due to voltage-dependent influx of  $\text{Ca}^{2+}$  are therefore associated with periodic activation of PLC ((Thore et al., 2004; Thore et al., 2007); Figure 3C,D). By generating  $\text{Ca}^{2+}$ -mobilizing  $\text{IP}_3$ , this mechanism provides a link between  $\text{Ca}^{2+}$  influx and release from intracellular stores that is distinct from classical CICR (Dyachok et al., 2004). Although  $\text{IP}_3$  is not essential for generating the slow  $[\text{Ca}^{2+}]_i$  oscillations,  $\text{IP}_3$ -mediated  $\text{Ca}^{2+}$  mobilization is important for their shaping (see above).

In addition to contributing to the generation and shaping of  $\text{Ca}^{2+}$  signals, PLC activation can be anticipated to regulate insulin secretion kinetics via generation of DAG and activation of PKC. Several conventional, novel and atypical PKC isoforms are expressed in islets and insulin-secreting cells (Jones and Persaud, 1998; Warwar et al., 2006). Activation of the PLC-PKC signalling pathway has been proposed to account for the second phase of glucose-induced insulin secretion (Zawalich and Zawalich, 1996). Conversely, insufficient activation of PLC may underlie both the desensitization of insulin release induced by prolonged exposure to high glucose and suppression of secretion by dexamethasone (Yamazaki et al., 2006a; Yamazaki et al., 2006b; Zawalich et al., 2006). Rapid changes in DAG formation have been detected in rat islets following stimulation with glucose or carbachol (Peter-Riesch et al., 1988; Wolf et al., 1989). Although DAG analogues and phorbol esters have been found to stimulate insulin secretion (Virji et al., 1978; Malaisse et al., 1980; Jones et al., 1985) by sensitizing the secretory machinery to  $\text{Ca}^{2+}$  (Jones et al., 1985; Tamagawa et al., 1985), it is debated whether PKC is involved in nutrient-induced insulin secretion (Metz, 1988; Jones and Persaud, 1998). The use of PKC inhibitors has resulted in conflicting conclusions (Jones and Persaud, 1998). Recent studies of PKC isoform-selective knock-out mice have indicated important roles for PKC $\delta$  and  $-\lambda$  in insulin secretion (Hashimoto et al., 2005; Uchida et al., 2007). Deletion of PKC $\epsilon$  did not affect normal glucose-induced insulin secretion, but amplified that in islets treated with fatty acids (Schmitz-Peiffer et al., 2007). However, another study showed that expression of a dominant negative mutant of PKC $\epsilon$  suppressed insulin exocytosis in isolated  $\beta$ -cells (Mendez et al., 2003). Some of the difficulties to define the role of PKC in insulin secretion may be due to the enzyme being transiently activated during the oscillatory PLC activity. Consistent with this idea, coordinated oscillations of

$[Ca^{2+}]_i$  and membrane translocation of PKC $\alpha$ , - $\beta$ II and - $\epsilon$  have been observed in both clonal and primary  $\beta$ -cells (Pinton et al., 2002; Zhang et al., 2004; Suzuki et al., 2006).

## 5. Cyclic AMP

Next to  $Ca^{2+}$ , cAMP is the most critical messenger for insulin secretion. cAMP is formed by adenylyl cyclases in response to  $G_s$ -coupled receptor agonists, such as glucagon, the incretin hormones glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP), as well as the neuropeptides pituitary adenylyl cyclase activating polypeptide (PACAP) and vasoactive intestinal polypeptide (VIP). Also glucose-stimulated islets show a modest elevation of cAMP (Charles et al., 1973; Grill and Cerasi, 1973; Hellman et al., 1974; Sharp, 1979), an effect that has been attributed to the elevation of  $[Ca^{2+}]_i$  (Charles et al., 1975; Valverde et al., 1979). From the observation that purified  $\beta$ -cells, lacking influence from the glucagon-producing  $\alpha$ -cells, show reduced cAMP content and impaired secretory capacity, it was suggested that cAMP has a permissive role in glucose-induced insulin release and that the effect of the sugar on cAMP content represents amplification of glucagon-induced cAMP production (Schuit and Pipeleers, 1985). Among several adenylyl cyclases expressed in islet cells and insulin-secreting cell lines (Leech et al., 1999; Guenifi et al., 2000; Delmeire et al., 2003), the  $Ca^{2+}$ -sensitive AC8 isoform has been proposed to integrate the cAMP responses to glucose and hormone receptor stimuli (Delmeire et al., 2003).

Early studies demonstrated that cAMP amplifies secretion in response to various initiators by promoting electrical activity and  $Ca^{2+}$  signals (Gylfe and Hellman, 1981; Henquin and Meissner, 1984; Eddlestone et al., 1985; Hellman et al., 1992), as well as by sensitizing the secretory machinery to  $Ca^{2+}$  (Tamagawa et al., 1985; Jones et al., 1986; Hellman et al., 1992).

The effect of cAMP on  $\text{Ca}^{2+}$  signals involves stimulation of both voltage-dependent  $\text{Ca}^{2+}$  entry and intracellular mobilization of the ion from the ER (Prentki et al., 1987; Prentki and Matschinsky, 1987; Grapengiesser et al., 1989a; Liu et al., 1996). While the former effect can be explained by PKA phosphorylation of voltage-dependent  $\text{Ca}^{2+}$  channels (Ämmälä et al., 1993a; Kanno et al., 1998) and  $\text{K}_{\text{ATP}}$  channels (Holz et al., 1993; Gromada et al., 2004), the intracellular mobilization is most likely due to PKA phosphorylation and sensitization of  $\text{IP}_3$  receptors (Liu et al., 1996; Dyachok and Gylfe, 2004). In addition to these mechanisms, cAMP has been reported to promote depolarization and intracellular  $[\text{Ca}^{2+}]_i$  mobilization via the cAMP-dependent guanine nucleotide exchange factor Epac (Kang et al., 2005; Kang et al., 2006), although another study questioned such effects (Dyachok and Gylfe, 2004).

The principal action of cAMP on exocytosis seems to be exerted at a step distal to the elevation of  $[\text{Ca}^{2+}]_i$  (Ämmälä et al., 1993a; Gillis and Mislser, 1993). The effects of cAMP on the exocytosis machinery involves both PKA-dependent and -independent mechanisms, the latter most likely mediated by Epac (Renström et al., 1997; Seino and Shibasaki, 2005). Many exocytosis-related proteins have been identified as substrates for PKA (Seino and Shibasaki, 2005), and the subcellular targeting of PKA to its effectors via A-kinase anchoring proteins has been found critical for the stimulatory effect of cAMP-elevating agents on insulin secretion (Lester et al., 1997; Fraser et al., 1998; Lester et al., 2001). Capacitance recordings have indicated that PKA mediates the slower cAMP-dependent mobilization of insulin granules, while Epac accounts for the rapid cAMP-dependent potentiation of exocytosis in  $\beta$ -cells (Renström et al., 1997; Eliasson et al., 2003). Epac has also been reported to increase the number of fusion sites (Kwan et al., 2007; Shibasaki et al., 2007) and along with PKA the number of granule-granule fusion events (Kwan et al., 2007). The effects of Epac are

mediated not only by its guanine nucleotide exchange activity on Rap1 (Shibasaki et al., 2007), but also by interactions with several other proteins, including the  $K_{ATP}$  channel regulatory subunit SUR1 (Ozaki et al., 2000; Eliasson et al., 2003), the voltage-dependent  $Ca^{2+}$  channel (Shibasaki et al., 2004) and the insulin granule proteins Rim2 (Ozaki et al., 2000) and Piccolo (Fujimoto et al., 2002).

Until recently, little was known about the spatiotemporal dynamics of cAMP signals in  $\beta$ -cells. However, the development of novel fluorescent reporters for cAMP in single cells have led to the demonstration that cAMP signals show complex temporal patterns in clonal insulin-secreting cells (Landa et al., 2005; Dyachok et al., 2006). Using a ratiometric evanescent wave microscopy approach, it was found that glucagon and GLP-1 triggered pronounced oscillations of cAMP beneath the plasma membrane of rat insulinoma cells (Dyachok et al., 2006). The hormone-induced cAMP elevations were synchronized with oscillations of  $[Ca^{2+}]_i$  (Fig.4). In MIN6-cells expressing a fluorescence resonance energy transfer biosensor, monophasic increases of cAMP was recorded in response to the GLP-1 receptor agonist exendin-4 as well as to glucose and depolarizing agents (Landa et al., 2005). When a high glucose concentration was combined with tetraethylammonium inhibition of  $K^+$  channels, there were fast oscillations of both  $Ca^{2+}$  and cAMP. In contrast to the GLP-1-induced cAMP oscillations (Dyachok et al., 2006), those evoked by tetraethylammonium were anti-synchronous with the oscillations of  $[Ca^{2+}]_i$ . This was interpreted as  $Ca^{2+}$ -dependent activation of the PDE1 family of phosphodiesterases (Landa et al., 2005). Islets and insulin-secreting cells express both  $Ca^{2+}$ -regulated phosphodiesterases (Pyne and Furman, 2003; Landa et al., 2005) and adenylyl cyclases (Leech et al., 1999; Guenifi et al., 2000; Delmeire et al., 2003), and among the cyclases certain isoforms are stimulated, whereas others are

suppressed by  $\text{Ca}^{2+}$  (Willoughby and Cooper, 2007). It is therefore likely that the phase relationship between cAMP and  $\text{Ca}^{2+}$  signals varies depending on the relative expression levels of different adenylyl cyclases and phosphodiesterases as well as on the type of stimulus (Fridlyand et al., 2007). The coordinated elevations and mutual enhancement of  $\text{Ca}^{2+}$  and cAMP signals observed in GLP-1-stimulated cells should be an exquisite trigger for exocytosis and may explain how the incretin hormone selectively enhances the pulsatile component of insulin release in healthy and diabetic subjects (Pørksen et al., 1998; Ritzel et al., 2001).

## **6. Feedback effect of insulin on secretion**

In addition to its endocrine effects leading to glucose uptake in muscle and adipose tissue and glycogen storage in liver, insulin has autocrine effects on  $\beta$ -cells, regulating gene transcription (Leibiger et al., 1998; Xu and Rothenberg, 1998; Wu et al., 1999; da Silva Xavier et al., 2000), proliferation (Withers et al., 1998; Kulkarni et al., 1999a; Okada et al., 2007), glucose metabolism (Borelli et al., 2004; Nunemaker et al., 2004), insulin biosynthesis and secretion (reviewed in (Rutter, 1999) and (Leibiger et al., 2002)).  $\beta$ -cells thus express insulin receptors (Verspohl and Ammon, 1980; Patel et al., 1982) as well as downstream adapter and signalling proteins, like insulin receptor substrate proteins IRS-1, -2, -3, -4, PI3-kinase and protein kinase B/Akt (Rothenberg et al., 1995; Velloso et al., 1995; Harbeck et al., 1996; Holst et al., 1998; Withers et al., 1998; Kulkarni et al., 1999b; Muller et al., 2006). Both exogenous insulin and endogenous glucose-induced insulin secretion have been found to activate insulin receptors, phosphorylate IRS-1 and activate PI3-kinase (Rothenberg et al., 1995; Velloso et al., 1995). PI3-kinase catalyzes the formation of 3'-phosphorylated

phosphoinositide lipids, in particular PIP<sub>3</sub>, and stimulation of islets and insulinoma cells with insulin or glucose is associated with increased levels of PIP<sub>3</sub> (Alter and Wolf, 1995; Idevall-Hagren and Tengholm, 2006; Yu et al., 2007). The presence of PIP<sub>3</sub> in non-stimulated  $\beta$ -cells (Alter and Wolf, 1995; Yu et al., 2007) has been taken to indicate that even basal insulin secretion exerts an autocrine effect (Yu et al., 2007). The generation of PIP<sub>3</sub> serves to recruit and activate signalling proteins to the plasma membrane. For example, via its PIP<sub>3</sub>-binding pleckstrin homology (PH) domain, protein kinase B/Akt is recruited to the membrane where it becomes phosphorylated by phosphoinositide-dependent kinase 1. In addition to its important role in regulating  $\beta$ -cell growth and survival (reviewed in (Dickson and Rhodes, 2004)), recent studies indicate that protein kinase B/Akt also regulates insulin granule exocytosis (Bernal-Mizrachi et al., 2004).

It is debated whether insulin stimulates, inhibits or does not influence insulin secretion (Leibiger et al., 2002). Arguments favouring a positive feedback effect include reduced insulin secretion from insulin receptor deficient mouse islets (Kulkarni et al., 1999a) and insulinoma cells (da Silva Xavier et al., 2004), increased insulin secretion in insulin-pretreated and IRS-1-overexpressing cells (Xu et al., 2000) as well as a direct stimulatory effect of insulin or an insulin-mimetic compound on secretion from individual cells (Aspinwall et al., 1999) or islets (Westerlund et al., 2002). Insulin is believed to exert its acute stimulatory effect via Ca<sup>2+</sup> mobilized from intracellular stores (Xu et al., 1999; Aspinwall et al., 2000; Xu et al., 2000). The underlying mechanism is not clear, but may involve IRS-1-mediated SERCA inhibition (Xu et al., 1999; Xu et al., 2000; Borge and Wolf, 2003) or generation of the Ca<sup>2+</sup> mobilizing messenger nicotinic acid adenine dinucleotide phosphate (Johnson and Mislser, 2002).



Negative feedback effects of insulin have been proposed based on observations that insulin or C-peptide secretion was suppressed by exogenous insulin *in vivo* (Elahi et al., 1982) or from the perfused pancreas (Iversen and Miles, 1971). More recent studies have demonstrated reduced insulin secretion from human islets following insulin receptor activation (Persaud et al., 2002), increased  $\text{Ca}^{2+}$  signalling and insulin secretion in insulinoma cells deficient in insulin receptors (Ohsugi et al., 2005), and insulin-induced hyperpolarization of  $\beta$ -cells (Khan et al., 2001). The latter effect likely involves PI3-kinase dependent formation of  $\text{PIP}_3$ , which activates  $\text{K}_{\text{ATP}}$  channels (Shyng and Nichols, 1998; Harvey et al., 2000). Additional support for negative feedback comes from observations that insulin secretion is stimulated by PI3-kinase inhibitors (Hagiwara et al., 1995; Zawalich and Zawalich, 2000) or genetic ablation of the PI3-kinase p85 regulatory subunit (Eto et al., 2002). The issue of insulin feedback effect is not yet settled and the reasons for the discordant results are not well understood. A recent study provides a possible solution to some of the inconsistencies by showing that low (<100 pM) insulin concentrations have a stimulatory effect, whereas high concentrations (>250 nM) have an inhibitory effect on C-peptide secretion from isolated islets (Jimenez-Feltstrom et al., 2004).

Considering the feedback effects of insulin it is obvious that pulsatile insulin release might generate oscillations in the insulin signalling pathway. Studies of the of PI3-kinase activity in MIN6 cells with an evanescent wave microscopy technique for monitoring plasma membrane concentrations of  $\text{PIP}_3$  recently revealed that glucose and insulin co-activate PI3-kinase and that glucose-induced insulin secretion is associated with pronounced oscillations of  $\text{PIP}_3$ , reflecting pulsatile insulin secretion from isolated cells (Idevall-Hagren and Tengholm, 2006); Fig. 5). Although the significance of the  $\text{PIP}_3$  oscillations remains

uncertain, their frequency and temporal relationship to  $[Ca^{2+}]_i$  oscillations are entirely consistent with an insulin feedback loop via PI3-kinase activation and formation of PIP<sub>3</sub>. In this way insulin may contribute to pulsatile insulin release by repolarizing glucose-stimulated  $\beta$ -cells (Khan et al., 2001; Idevall-Hagren and Tengholm, 2006).

## 7. Conclusions and future perspectives

It is obvious that pulsatile insulin secretion is controlled by many different factors at multiple organizational levels. Importantly, the individual  $\beta$ -cell possesses an inherent ability to generate oscillatory signals that underlie pulsatile release of insulin. Recent studies have demonstrated that oscillations of  $[Ca^{2+}]_i$  are coordinated with oscillations in  $\beta$ -cell metabolism, PLC activity as well as intracellular cAMP and plasma membrane phosphoinositide lipid concentrations. There are complex interdependencies between the different messengers and signalling pathways that contribute to the amplitude and shape of the insulin secretory response to nutrients and neurohormonal factors. Several of these pathways may be important targets for pharmacological treatment of type 2 diabetes. Interestingly, both sulphonylureas and GLP-1 treatment improves in vivo pulsatility (Pørksen, 2002), which may be related to the ability of the drugs to generate oscillations of  $[Ca^{2+}]_i$  (Grapengiesser et al., 1990) and cAMP (Dyachok et al., 2006). Impairment of insulin secretion from the  $\beta$ -cells is central in the development of type 2 diabetes, but the perturbation of in vivo insulin pulsatility in diabetes does not necessarily reflect a  $\beta$ -cell defect, since it might result from impaired synchronization of the secretory activity of  $\beta$ -cells within or between the many islets in the pancreas.

The significance of pulsatile insulin secretion is mostly discussed with regards to the action of the hormone on the peripheral target tissues, in particular the liver (Pørksen, 2002). However, pulsatile insulin release may also be important at the level of the individual  $\beta$ -cell. In view of the rapid and strong autocrine activation of insulin receptors in the  $\beta$ -cell, its insulin signalling machinery might be expected to be strongly and constitutively activate, if not desensitized, if the cells were exposed to extended periods of high insulin concentrations. In contrast, pulsatile insulin release is associated with periods without or with only low intensity autocrine stimulation, which enables the cells to sense the background concentration of circulating insulin and other growth factors crucial for  $\beta$ -cell function. Since insulin has been suggested to be an important stimulus for  $\beta$ -cell proliferation in states of insulin resistance (Okada et al., 2007), loss of pulsatile insulin secretion in prediabetic and diabetic states (Lang et al., 1981; O'Rahilly et al., 1988) may not only be envisioned to contribute to insulin resistance in the extrapancreatic target tissues, but also to impair the compensatory expansion of the  $\beta$ -cell mass.

Future studies will have to clarify the significance of oscillations in different  $\beta$ -cell signalling pathways as well as the molecular mechanisms underlying their generation. Given the spatio-temporal complexity of cellular signalling, progress in this area will rely on the continued development of tools and technologies for tracking signalling events in individual cells. A combination of real-time recordings of signalling events with traditional biochemical approaches, molecular perturbation strategies and mathematical modelling is required to improve our understanding of oscillatory control of insulin secretion.

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Table 1. Characteristics of different types of  $[Ca^{2+}]_i$  oscillations in single  $\beta$ -cells and isolated pancreatic islets.

Type of $[Ca^{2+}]_i$ oscillation	Preparation	Frequency	Duration of each $[Ca^{2+}]_i$ increase	Glucose dependence	Dependence on voltage-operated $Ca^{2+}$ influx	Dependence on intracellular $Ca^{2+}$ uptake and release	Main stimuli
Slow	single $\beta$ -cells	0.1-0.5 $min^{-1}$	1-5 min	threshold for induction	essential	not required, but shaping effect	glucose, leucine, tolbutamide
Slow	islets	0.15-0.3 $min^{-1}$	1-5 min	threshold for induction	essential	not required, but shaping effect	glucose, leucine, tolbutamide
Fast	islets	1-5 $min^{-1}$	<40 s	strong effect on duration above threshold	essential	some dependence	glucose
Spiking	single $\beta$ -cells and islets	often irregular	<40 s	graded above threshold	not required, but stimulating	essential, mediated by $IP_3$ and cAMP	cAMP-elevating agents
Fast receptor-induced	single $\beta$ -cells and islets	1-6 $min^{-1}$	<40 s	graded	independent	essential, mediated by $IP_3$	phospholipase C-activating receptor agonists

### Figure legends

Fig. 1. Pulsatile insulin secretion at different organizational levels. The upper panel shows a recording of insulin concentration in the portal vein of the perfused rat pancreas (adopted from Salehi et al 2005). Pulsatile insulin secretion from the pancreas requires coordination of the secretory activity of the individual islets via intrapancreatic nerves and diffusible factors. The middle panel shows insulin secretion from an isolated pancreatic islet (adopted from Bergsten et al., 1994). The synchronization of the oscillations in individual  $\beta$ -cells via gap junctions and diffusible factors is a prerequisite for pulsatility at this level. The bottom panel illustrates pulsatile insulin secretion from an individual  $\beta$ -cell recorded with a fluorescent biosensor for autocrine activation of insulin receptors (Idevall-Hagren & Tengholm, 2006).

Fig. 2. Cytoplasmic  $\text{Ca}^{2+}$  oscillations and electrical activity in individual  $\beta$ -cells. (A) Elevation of the glucose concentration from 3 to 11 mM results in a small initial lowering followed by a pronounced rise and oscillations of  $[\text{Ca}^{2+}]_i$  with a period of 2-10 min in a single mouse  $\beta$ -cell as measured with the  $\text{Ca}^{2+}$  indicator fura-2. (B) In addition to the slow  $[\text{Ca}^{2+}]_i$  oscillations, glucose triggers rapid  $[\text{Ca}^{2+}]_i$  spikes superimposed on the slow oscillations. These spikes become more frequent after exposure of the cells to cAMP-elevating agents. The traces show  $[\text{Ca}^{2+}]_i$  recordings with fura-2 from two separate cells exposed to 20 mM glucose and 20 nM glucagon (modified from Grapengiesser et al., 2004). It is notable that both the slow oscillations and the spikes are synchronized between the cells, although they are not in physical contact. (C) The slow  $[\text{Ca}^{2+}]_i$  oscillations correlate with bursts of action currents in a single mouse  $\beta$ -cell exposed to 11 mM glucose. The top trace shows action currents recorded with the cell-attached configuration of the patch-clamp technique. The middle trace show the

frequency of the action currents and the lower trace show  $[Ca^{2+}]_i$  measured with fura-2. (D) Rapid spikes of  $[Ca^{2+}]_i$  are associated with interruptions of the action currents, probably as a result of the activation of a hyperpolarizing current. (C) and (D) are adopted from Dryselius et al. (1999).

Fig. 3. Dynamic single-cell recordings of phospholipase C activity using evanescent wave fluorescence microscopy. (A) Fusion of GFP with the PIP<sub>2</sub>- and IP<sub>3</sub>-binding PH domain from PLC $\delta$ , generates a biosensor that is associated with PIP<sub>2</sub> in the plasma membrane under basal conditions and which dissociates when this lipid is hydrolyzed by PLC and the concentration of IP<sub>3</sub> increases in the cytoplasm. In evanescent wave microscopy, fluorescent molecules are excited within ~100 nm from the plasma membrane by total internal reflection of a laser beam at the interface between a cover slip and the aqueous medium bathing the adherent cells. Fluorescent biosensor binding to the plasma membrane is associated with bright fluorescence, whereas the translocation to the cytoplasm is recorded as loss of fluorescence. (B) Schematic drawing of the control of PLC activity by Ca<sup>2+</sup>. Agonist (A) activation of a G-protein coupled receptor (GPCR) results in partial activation of PLC via the Gq family of G-proteins. The resulting hydrolysis of PIP<sub>2</sub> leads to formation of diacylglycerol (DAG) and sufficient amounts of IP<sub>3</sub> to mobilize Ca<sup>2+</sup> via IP<sub>3</sub>-receptors (IP<sub>3</sub>R) from the ER. The subsequent elevation of  $[Ca^{2+}]_i$  feedback activates PLC, which generates more IP<sub>3</sub> and so forth. The reduced luminal Ca<sup>2+</sup> concentration in the ER activates store-operated channels (SOC) in the plasma membrane and the resulting influx of Ca<sup>2+</sup> stimulates PLC activity during sustained receptor stimulation. PLC can also be directly activated by membrane depolarization (depol) and Ca<sup>2+</sup> influx via voltage-dependent Ca<sup>2+</sup> channels (VOC). (C)

Simultaneous measurements of  $[Ca^{2+}]_i$  (black) and membrane  $PIP_2$  concentration (green) in a MIN6 cell using the  $Ca^{2+}$  indicator Fura Red and the biosensor PLC $\delta$ PH-GFP. Elevation of glucose triggers oscillations of  $[Ca^{2+}]_i$  that coincides with loss of  $PIP_2$  (activation of PLC). Adopted from Thore et al. (2007). (D) Glucose does not affect PLC activity when voltage-dependent  $Ca^{2+}$  influx is prevented by the hyperpolarizing agent diazoxide. Removal of this agent immediately leads to translocation of the biosensor, reinforcing the importance of  $Ca^{2+}$  for glucose activation of PLC.

Fig. 4. GLP-1-induced cAMP oscillations in single insulin-secreting cells measured with a fluorescent translocation reporter and ratiometric evanescent wave microscopy. Increases of reporter fluorescence ratio correspond to increases in cAMP concentration. (A) Stimulation of INS-1 cells with 1 nM GLP-1 in the presence of 3 mM glucose evokes oscillations of cAMP that disappears upon removal of extracellular  $Ca^{2+}$ . (B) Simultaneous recording of cAMP (green) and  $[Ca^{2+}]_i$  (black) in a single INS-1 cell reveals that the GLP-1-induced cAMP oscillations are perfectly synchronized with oscillations of  $[Ca^{2+}]_i$ . Adopted from Dyachok et al. (2006).

Fig. 5. Glucose-induced oscillations of plasma membrane  $PIP_3$  concentration. Evanescent wave microscopy recordings from individual MIN6 cells expressing a  $PIP_3$ -binding GFP construct (GFP $_4$ -GRP1) that translocates to the membrane upon PI3-kinase-catalyzed formation of the lipid. This translocation is recorded as an increase of fluorescence. (A) Elevation of the glucose concentration from 3 to 11 mM glucose triggers pronounced  $PIP_3$



oscillations. (B) The glucose effect on  $\text{PIP}_3$  depends on feedback from secreted insulin and is consequently prevented by blocking secretion with the hyperpolarizing agent diazoxide.

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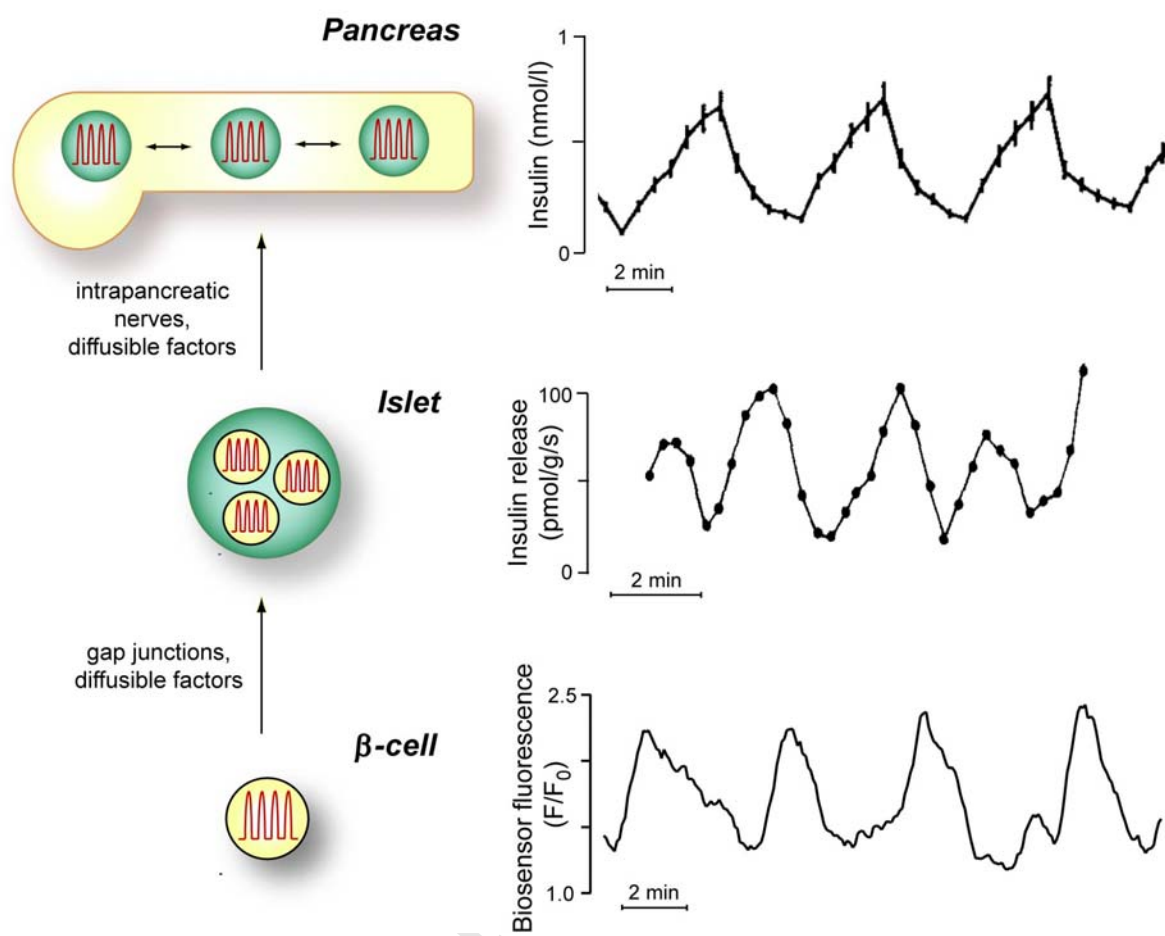


FIG. 1

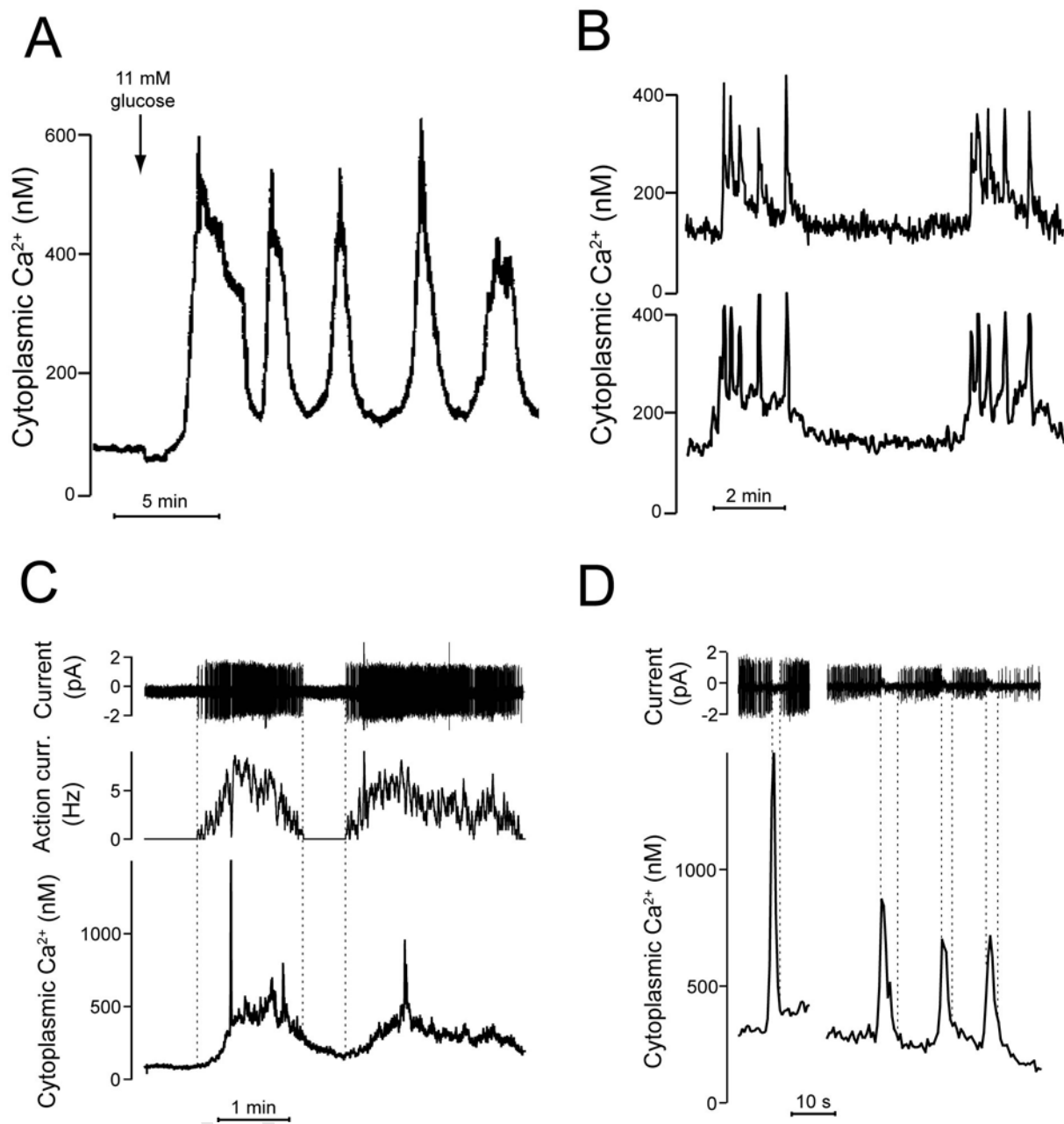


FIG. 2

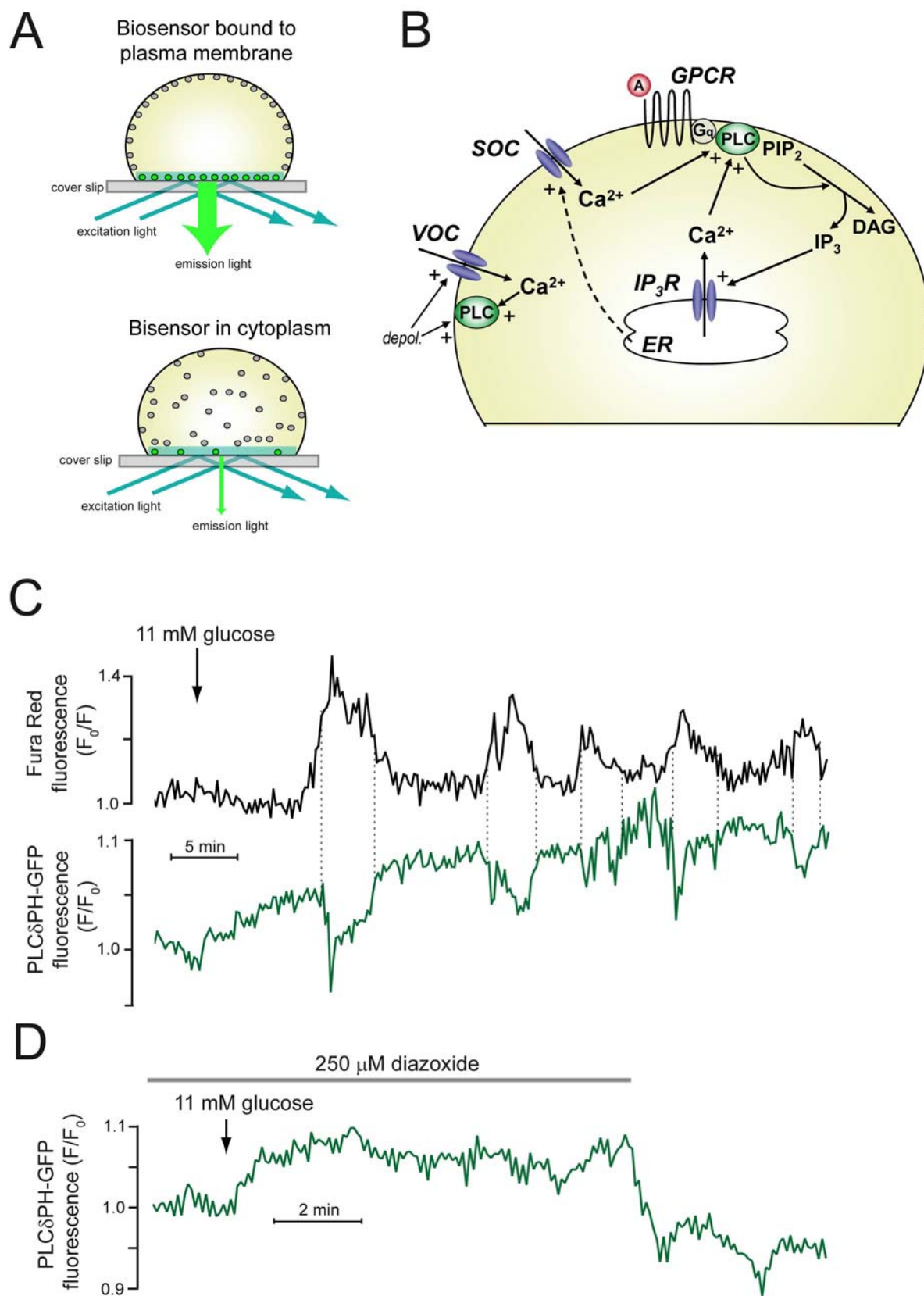


FIG. 3

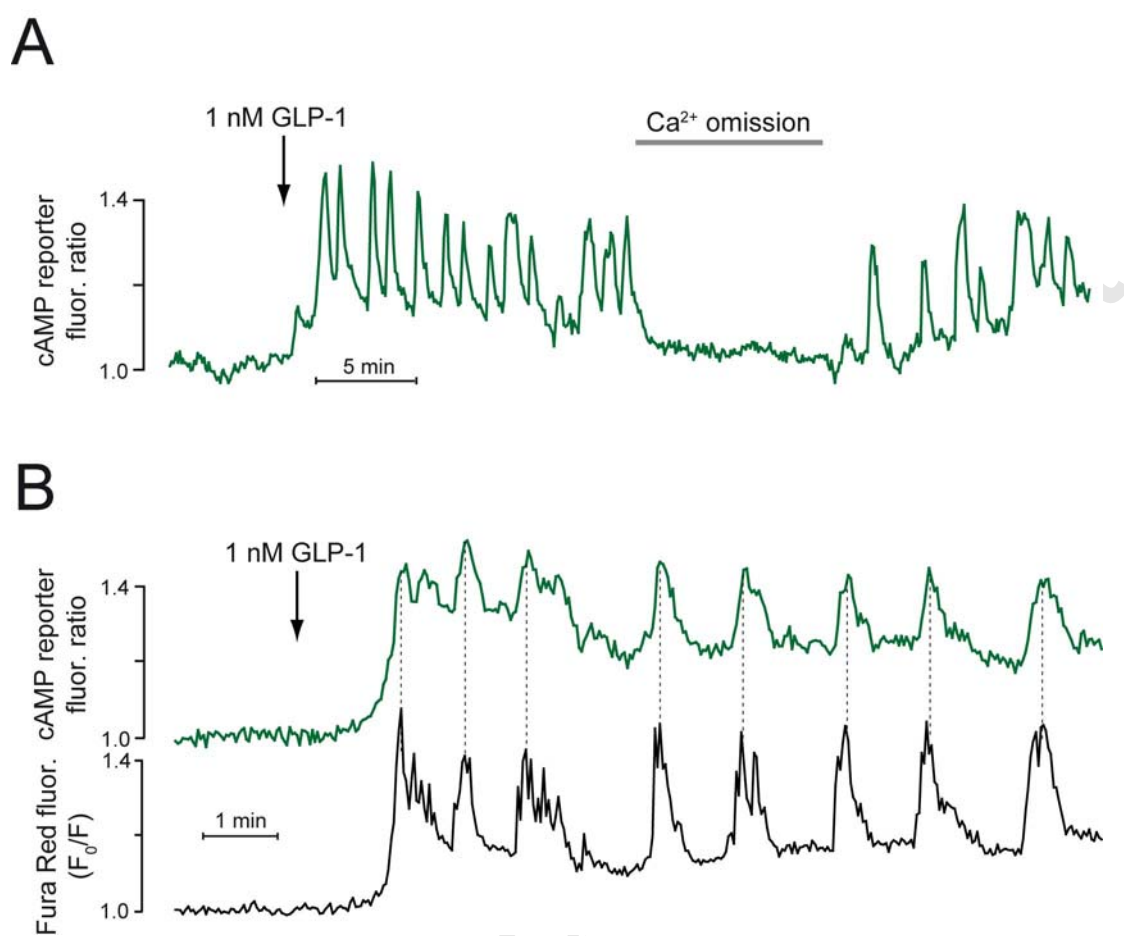


FIG. 4

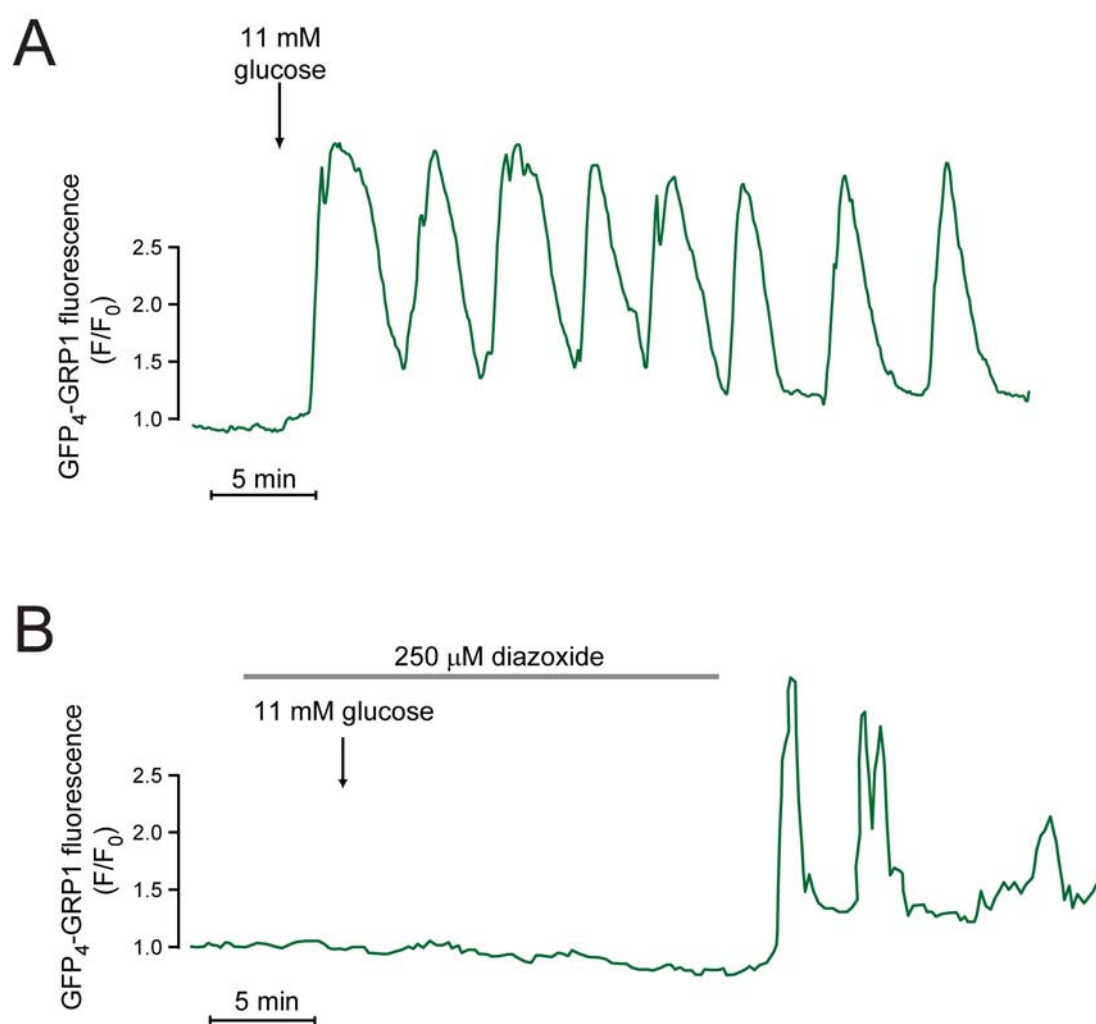


FIG. 5