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MECHANISMS OF ACTION OF NONGLUCOSE INSULIN SECRETAGOGUES

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INTRODUCTION

Insulin plays a crucial role in maintaining blood glucose in the physiologic range. Insulin secretion is regulated by several factors. The physiological insulin secretagogues fall into two categories: nutrient secretagogues and non-nutrient secretagogues. In the group of nutrient secretagogues, only glucose

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may initiate insulin release. Amino acids and fatty acids can also stimulate insulin release but usually require the presence of basal glucose levels in order to do so. Nonnutrient insulin secretagogues, which do not initiate but merely modulate the secretory response, can be divided into three subclasses: neurotransmitters, hormones, and various synthetic chemical compounds, e.g. hypoglycemic drugs. These secretagogues may affect β-cells through the following processes: (*a*) Some nonnutrient secretagogues, such as hormones and neurotransmitters, bind to specific receptors of the β-cell membrane and activate various second messenger systems. Second messengers may change protein phosphorylation and phospholipid metabolism, which in turn may alter $[Ca^{2+}]_i$ level and trigger insulin secretion. (*b*) Hypoglycemic drugs such as sulphonylurea compounds change the activity of ion channels in the β-cell membrane, alter the membrane potential, and increase $[Ca^{2+}]_i$. This increase triggers exocytosis of the insulin-containing secretory granules. Basal glucose is necessary to activate the stimulation-secretion process by the majority of nonglucose secretagogues. Although it is the purpose of this review to summarize the recent advances in the mechanism(s) of nonglucose insulin secretagogues, the role of glucose metabolism in insulin release must first be elucidated, because it provides the foundation for understanding the mechanism(s) of nonglucose secretagogues. We then discuss four aspects of this mechanism(s): receptor activation, the cAMP system, lipid-derived messengers, and ion channel regulation.

GLUCOSE AS PRIMARY β-CELL STIMULUS

Glucose plays a dual function in the pancreatic β-cell in that it is both a fuel and a physiological stimulus for insulin secretion. Glucose-induced insulin release is tightly dependent on the regulation of metabolic events in the pancreatic islet.

The main route for glucose metabolism in islets is glycolysis (64). Glycolysis and the subsequent oxidation of pyruvate via the Krebs cycle are of crucial functional importance for control of insulin secretion. For such a mechanism to be functional in the β-cell, the system requires a device to translate changes in the blood glucose concentration into corresponding signal-generating metabolic flux rates for initiation of insulin secretion. This device is composed of the glucose transporter (GLUT2) and glucokinase. GLUT2 transports glucose into β-cells by facilitated diffusion. This glucose transporter is characterized by a glucose K_m of 15–20 mM and a V_{max} of 32 mmol/min per liter of cellular H_2O (47), a rate almost 100 times faster than that of glucose phosphorylation catalyzed by glucokinase (100). With this high capacity of glucose transport, the intracellular glucose concentration in the β-cell is similar to the extracellular concentration. However, a decrease of GLUT2 at both the mRNA and

protein levels has recently been found in several rodent models of both insulin-dependent diabetes mellitus and non-insulin-dependent diabetes mellitus. This decrease was associated with a reduced glucose uptake rate and glucose-induced insulin release (16, 80, 81). Therefore, it has been proposed that GLUT2 dysfunction may play an important role in the pathogenesis of diabetes (102).

To further test this hypothesis, we used quantitative histochemical techniques to measure islet glucose uptake in situ in several animal models of diabetes or obesity, all of which had reduced glucose-induced insulin secretion (55). The basal glucose content in the islets of the *db/db* mouse, Bureau of Home Economics (BHE) rat (8), partially pancreatectomized rat (12), and *fa/fa* Zucker rat is higher than in normal animals as a result of hyperglycemia, which indicates that under steady-state conditions, glucose uptake was not limiting in these islet cells. After glucose injection, the glucose uptake rate in the first minute was significantly slower in the *db/db* mice and *fa/fa* Zucker rats than in their respective controls. Conversely, no reduction of glucose uptake occurred in the BHE and pancreatectomized rats. A RIP-cHras transgenic mouse has a 90% reduction of GLUT2 in islets, but the glucose-induced insulin release in perfused pancreas was similar to that from normal mouse. We also measured the islet glucose uptake in situ in these transgenic mice and found no significant change in the glucose uptake rate. In the GK rat, a non-insulin-dependent diabetes mellitus model (87), much less insulin was released by glucose compared with the decrease of GLUT2 level in pancreatic islets (77). In summary, GLUT2 may be a rate-limiting step in acute insulin release stimulated by glucose in some diabetic models; however, it is not likely a universal cause in the pathogenesis of diabetes. Insulin and glucose may regulate GLUT2 expression at the mRNA and protein levels. Whether GLUT2 is regulated at the posttranslational and translocational levels is not yet clear.

As soon as glucose enters the β-cell, glucose metabolism begins with glucose phosphorylation by glucokinase. Glucokinase functions as a pacemaker of glucose metabolism in pancreatic β-cells (62, 64). It has a glucose K_m of 5–10 mM and a Hill number of 1.5 (63, 72). These parameters determine the inflection point of this enzyme, which is around 4–5 mM glucose. Under physiological condition, any change in the blood glucose, however small, will effectively alter the rate of glucose phosphorylation and, in turn, regulate the rate of glucose metabolism in islet. Islet glucokinase is regulated by glucose rather than insulin, whereas liver glucokinase is induced by insulin (6, 46, 56). This contrast is due to different promoters of the glucokinase gene that are operative in islet and in liver (59). The basis of glucose induction of β-cell glucokinase remains unclear. Glucose does not likely act at the transcriptional level, since no significant changes of glucokinase mRNA were observed when the ambient glucose was changed both in vivo and in vitro (46, 56). Whether

glucose regulates glucokinase translationally or posttranslationally is still under investigation. In addition to glucose, a regulatory protein may modify islet glucokinase activity (105). This regulatory protein was identified in liver, has a M_r of 62.9 kDa (20), and is activated by fructose 6-phosphate. The active form of this regulator can bind to liver glucokinase and decrease the affinity of glucose to glucokinase (106). This glucokinase regulatory protein may also exist and function in islets (61).

Recently, more than 20 mutations in the glucokinase gene were found in patients with maturity onset diabetes of the young (7, 30). These mutants of glucokinase have either an increased glucose or ATP K_m or reduced phosphorylating activity (33, 99; Liang, Wang, Kesavan, Magunson, Matchinsky, et al, unpublished data]. This important discovery strongly supports the view of glucokinase as the glucose sensor in β-cells and highlights the study of the mechanism of glucose-induced insulin release.

In addition to glucokinase, other factors may also regulate glucose metabolism and affect insulin release. Control of glucose phosphorylation is complicated by the presence of glucose 6-phosphatase, a microsomal enzyme that may lead to futile cycling between D-glucose and D-glucose 6-phosphate (107). In islet, this enzyme may be important for the regulation of the glucose 6-phosphate concentrations because it accounts for as much as 40% of the rate of glucose phosphorylation in intact islets, as reported in the *ob/ob* mouse (50). Decreased activity of islet FAD-linked glycerophosphate dehydrogenase was recently found in the GK rat (82) with a deficient oxidative metabolism of glucose in islet mitochondria (34). This same defect was also observed in the *db/db* mouse (94), which indicates that the glycerol phosphate shuttle in islet may also regulate the process of glucose-stimulated insulin release.

G PROTEINS, CYCLIC AMP, AND INSULIN RELEASE

In addition to blood glucose, neurotransmitters and hormones regulate insulin release under physiological condition. Acetylcholine and catcholamines are important neurotransmitters that alter insulin secretion. Hormones affecting insulin release are derived from islet non-β-cells (e.g. somatostatin) or from the intestine (e.g. glucagon-like peptide, gastric inhibitory polypeptide). These neurotransmitters and hormones first bind to specific receptors on the β-cell membrane and then generate second messengers that regulate insulin release.

G Protein Couples the Receptor to Adenylyl Cyclase

Guanine nucleotide-binding proteins (G proteins) are essential in stimulus-secretion coupling in the pancreatic islets. They link hormone receptors to signal-transduction systems, e.g. adenylyl cyclase, ion channels, phospholipases, and distal sites in exocytosis in the pancreatic β-cells.

G proteins are heterotrimers composed of α, β, and γ subunits, which on activation dissociate into the α-guanine nucleotide monomer and the β and γ subunits. The α subunits of M_r from 39 to 50 kDa bind and hydrolyze guanosine triphosphate (GTP), define the receptor and effector specificity of a G protein, and differ from G protein to G protein. The α subunits of all G proteins are substrates of either cholera toxin or pertussis toxin, both of which function as ADP-ribosyltransferase. G proteins are divided into several subtypes according to their function. G_s has been established as the stimulatory regulatory component of adenylyl cyclase and as the component responsible for high-affinity binding of hormones to adenylyl cyclase stimulatory receptors. G_s function is inhibited by cholera toxin. G_i is a family of proteins modified by pertussis toxin, which blocks not only hormonal inhibition of adenylyl cyclase, but also hormonal stimulation of arachidonic acid release from cells such as macrophages as well as some hormonal stimulation of the phospholipase C pathway. G_E may be a regulator in the pathway leading to fusion of secretory vesicles with the plasma membrane.

The favored theory explaining activation of G protein involves a critical exchange of GTP for GDP on the α-subunit catalyzed by the interaction of the heterotrimeric G protein with the hormone-receptor complex. This association is followed by a GTP-GDP exchange, whereupon the β- and γ-subunits dissociate from the heterotrimeric complex, allowing the dissociated α-subunit-GTP complex to enter an activated state. It is this activated species that associates with the effector systems (e.g. adenylyl cyclase) and regulates effector-system function. The activated α-subunit possesses intrinsic GTPase activity and hydrolyzes the bound GTP, which terminates activation of the α-subunit. Thus closely integrated activation and autodeactivation of the G-protein α-subunit completes the cycle and allows the α-subunit-GDP complex, reassociated with β- and γ-subunits, to re-form heterotrimers and return to the quiescent state.

Several types of G protein, such as $G_{s\alpha}$, G_i ($G_{i\alpha1}$, $G_{i\alpha2}$, $G_{i\alpha3}$, and G_{i2}), and G_O (G_{O1} and G_{O2}) (10, 11, 92), have been demonstrated by ADP ribosylation and Western blotting in pancreatic islet as well as in HIT T15 cells. Extensive data support the concept of G_i as a β-cell physiological regulatory protein. This view is based on the effects of epinephrine (111), somatostatin (40, 111), prostaglandin E_2 (93), and galanin (95). To the extent that cAMP augments glucose-induced insulin secretion, G_s also appears to be an important modulatory factor of physiologically stimulated β-cell function. An example of G_s protein involving insulin release through cAMP is the glucagon-like peptide (GLP), a recently discovered hormonal mediator of the entero-insular axis. GLP augments nutrient-induced insulin secretion and is generated by post-translational processing of proglucagon in the small intestine. Following oral ingestion of food, GLP is secreted into circulation. In humans, infusion of GLP

induces a clear elevation of insulin release (53). Studies with several insulinoma cell lines and with isolated rat islets have revealed the presence of a specific GLP receptor (101) containing eight hydrophobic segments, seven of which are most likely transmembrane domains. The first amino terminal hydrophobic segment represents a leader sequence. It belongs to the superfamily of heterotrimeric G protein–coupled receptors. GLP binds to the receptor and activates adenylyl cyclase via a presumed guanine nucleotide-binding protein–coupling step (27). The increase in cAMP content converts latent voltage-dependent calcium channels into operative channels (58). The influx of Ca^{2+} through voltage-dependent calcium channels plays a key role in the potentiation of glucose-stimulated insulin secretion by GLP.

One group of G proteins stimulates phospholipase activity by regulating three phospholipases: A_2, C, and D. A direct link between a G protein and phospholipase A_2 was suggested by the finding that stimulation of phospholipase A_2 is pertussis toxin–sensitive (13) and that fluoride, an activator of G proteins, can stimulate arachidonic acid release in intact or permeabilized cells (71). Phospholipase C_β has been identified in association with the cell membrane (18). This β isoform is responsible for the stimulation of GTPγS or fMetLeuPhe in HL60 cell membranes (96). The fact that the fMetLeuPhe response in membranes can be inhibited by pertussis toxin treatment implies that phospholipase C_β can couple to pertussis toxin–sensitive G proteins. GTPγS-stimulated phospholipase D activity has also been demonstrated in membrane fractions in several cell types. The effect of these phospholipases on insulin release is discussed in a later section. G_E may play a role in nonglucose secretagogue-induced insulin release; however, no detailed research data are available at present.

Cyclic AMP: The Second Messenger in Hormone-Stimulated Insulin Release

In β-cells, cAMP is elevated by glucose and hormones such as glucagon, GLP, and gastric inhibitory polypeptide (GIP). An increase in cAMP, induced by nonglucose secretagogues, magnifies the secretory response to glucose stimulation. An increase in cAMP concentration in pancreatic β-cells produces at least three important effects in these cells: It regulates insulin gene transcription, increases $[Ca^{2+}]_i$ and/or modifies sensitivity to $[Ca^{2+}]_i$, and initiates rapid phosphorylation of proteins. Cyclic AMP–dependent protein kinase A plays a crucial role in this process.

Cyclic AMP can regulate insulin biosynthesis at the transcriptional level. Philippe & Missotten (86) analyzed the promoter region of the rat insulin I gene and identified a cAMP-response element through which cAMP may regulate gene transcription. They also identified a 43 kDa nuclear protein in HIT cells that binds specifically to the cAMP-response element. Phosphory-

lation of this cAMP-response element–binding protein by protein kinase A may affect transcription of the insulin gene in rat islets. It has been reported that forskolin, a adenylyl cyclase activator, significantly increases proinsulin mRNA levels in insulinoma β-TC-1 cell (28).

High levels of cAMP in pancreatic β-cell also induced an increase of $[Ca^{2+}]_i$ through activation of L-type Ca^{2+} channels. For example, forskolin stimulated electrical activity and ion fluxes in mouse pancreatic islets (38). Increases in $^{45}Ca^{2+}$ and in $^{86}Rb^{2+}$ efflux induced by forskolin in the presence of glucose were prevented by verapamil or by reducing the medium Ca^{2+} concentration. However, Eddlestone et al (26) demonstrated that forskolin stimulates biphasic electrical activity in mouse pancreatic islets even at substimulatory glucose concentrations. A possible explanation for this finding is that elevation of cAMP either reduced K^+ permeability by phosphorylation of a Ca^{2+}-sensitive K^+ channel or altered the gating of Ca^{2+} channels in the β-cell membrane. This hypothesis is feasible because both Ca^{2+}-sensitive K^+ channels and voltage-sensitive Ca^{2+} channels may be targets of cAMP-dependent protein kinase A (38, 2).

An additional mechanism that has been postulated to explain the potentiation of insulin release by cyclic AMP involves sensitization of the secretory mechanism to Ca^{2+}. In permeabilized pancreatic islets or RINm5F cells, intracellular Ca^{2+} concentrations were held constant by inclusion of EGTA, and the addition of cAMP, IBMX, or forskolin augmented Ca^{2+}-induced insulin release (48, 104). These agents also promoted a shift in the dose-response curve of the Ca^{2+}-induced insulin release to lower Ca^{2+} concentrations. All of these studies suggested that cAMP stimulates insulin release (at least in part) by sensitizing the secretory mechanism to Ca^{2+}. The fact that forskolin modestly stimulated insulin release in the presence of glucose but in the absence of extracellular Ca^{2+} also supports this concept. In HIT cells loaded with quin 2, Hughes et al found that forskolin potentiated glucose-induced insulin release at low (0.1 mM) extracellular Ca^{2+} concentrations in which no apparent change in $[Ca^{2+}]_i$ was observed (43). It has been suggested that cAMP may directly interact with the secretory machinery of β-cells, an effect that may account for as much as 80% of the cAMP effect (2). The mechanism by which protein kinase interacts with and sensitizes the secretory machinery to Ca^{2+} at the molecular level remains to be defined.

The potentiation of insulin secretion by cAMP is accompanied by rapid phosphorylation of specific islet substrates of protein kinase A. Although protein kinase A has been detected and characterized in β-cell (97), its protein substrates have not been clearly identified. Studies involving intact islets or subcellular fractions of islet cells have identified as many as 15 peptides phosphorylated by protein kinase A (42). For example, Christie & Ashcroft (17) prelabeled intact islets for 1 h with ^{32}Pi and stimulated them with forskolin

to elevate intracellular cAMP. Subcellular fractionation revealed the presence of four major phosphorylated peptides: two cytosolic phosphopeptides with M_r of 30 and 25 kDa, respectively, and two particulate phosphopeptides with M_r of 32 and 23 kDa, respectively. The 23 kDa particulate phosphopeptide was located in the secretory granule fraction, whereas the 32 kDa particulate phosphopeptide appeared to be ribosomal in origin. However, the nature and subcellular locations of these protein kinase A substrates were not further characterized. The correlation of the phosphorylation status of protein kinase A substrates with insulin release must be further explored in order to identify the key peptide substrates involved in the secretory process.

PHOSPHOLIPIDS AND INSULIN RELEASE

Phospholipids play an important role in the cellular signaling of insulin release stimulated by several nonglucose secretagogues. Three types of phospholipases—phospholipase A_2, C, and D—have been found in islet cells (66). The presence of phospholipase A_2 was confirmed by identifying its enzymatic activity, which releases arachidonic acid or generates another specific product of phospholipase A_2, i.e. 1-fatty acyl, 2-lysophosphatidylcholine. The presence of mRNA for type I phospholipase A_2 in intact islets and HIT cells further substantiated the presence of this enzyme in β-cells (68). Immunocytochemical studies have found phospholipase C in the islet, specifically in β-cells (21, 110). Assessment of the accumulation of the hydrolytic end products or of the transphosphatodylation product enabled the identification of phospholipase D in rat islets. Phospholipase D activity was also observed in human islets (66).

Inositol 1,4,5-Triphosphate

Rat islets possess an active transport mechanism for inositol accumulation. This mechanism is independent from the glucose-uptake mechanism and is probably driven by the transmembrane Na^+ gradient, since inositol accumulation is inhibited under conditions when this gradient is compromised. One target of intracellular inositol in the β-cell is the membrane phospholipid pool, where it can be readily incorporated into phosphatidylinositol and the polyphosphoinositides. Islet cell plasma membranes are home to a polyphosphoinositides kinase activity, which phosphorylates polyphosphoinositides to yield phosphatidylinositol 4-phosphate. This enzyme is inhibited by Ca^{2+} over the range of 1–10 μM, which indicates that its activity may be regulated by stimulus-induced increases in cytosolic-free Ca^{2+} as part of a feedback control mechanism (91). A second kinase activity capable of catalyzing the formation of phosphatidylinositol 4,5-bisphosphate takes place in β-cell plasma membranes. A calcium-activated phosphomonoesterase is responsible for phosphatidylinositol 4,5-bis-phosphate degradation (23). Membrane inositol- phos-

pholipids were hydrolyzed by phospholipase C to inositol triphosphate (IP_3) and diacylglycerol (DAG). Muscarinic agonists such as carbachol stimulate phospholipase C activity in pancreatic β-cells and result in the accumulation of IP_3 and DAG. Activation of phospholipase C by carbachol is accomplished through G protein (22) and is dependent on the presence of Ca^{2+}.

The IP_3 released from phosphatidylinositol 4,5-bis-phosphate promotes a rise in the cytosolic-free Ca^{2+} concentration by inducing endogenous Ca^{2+} mobilization. This process is caused by the release of Ca^{2+} from internal Ca^{2+} pools, which are IP_3 sensitive. Increase in $[Ca^{2+}]_i$ triggers exocytosis, which is discussed at a later point in the chapter.

Diacylglycerol

Another consequence of phospholipase C–catalyzed phospholipid breakdown is the generation of DAG, which is considered an important signal molecule in the pancreatic β-cells for several reasons. First, increased production of DAG appears to increase the fusogenic potential of biological membranes, which may be important for exocytosis. Second, DAG serves as a substrate for DAG lipase, an enzyme involved in the liberation of arachidonic acid from the *sn*-2 position of the molecule. Third and most important, DAG activates protein kinase C.

The major isoenzymes of protein kinase C in rat pancreatic islets are α- and β_{II}, identifiable by immunohistochemical staining and Western blot using specific antibodies (45, 31). When the enzyme exists in the cytoplasm, a pseudosubstrate is thought to bind to the substrate-binding site, rendering the kinase inactive. Binding of DAG supposedly produces a conformational change that results in dislocation of the pseudosubstrate from the active site, an event that activates the kinase. Protein kinase C activation is often reflected by translocation from cytosol to membranes. It is demonstrated that muscarinic agonist–induced DAG accumulation in islets activates protein kinase C, and this process is involved in amplifying glucose-induced insulin secretion by muscarinic receptor agonists. This was drawn from: (*a*) Carbachol increases the DAG level in rat islet (85); (*b*) in rat islet homogenates, DAG causes a dose-related activation of protein kinase C (60); (*c*) carbamylcholine can induce translocation of protein kinase C activity from cytosol to membranes of pancreatic islets (84) and stimulates myristoylated alanine-rich C kinase substrate (MARCKS) phosphorylation (25); (*d*) prolonged exposure of islets to 12-*O*-tetradecanoylphorbol-3-acetate depletes protein kinase C activity and suppresses amplification of glucose-induced insulin release by carbamylcholine (83); and (*e*) the protein kinase C inhibitor staurosporine suppresses both carbachol-induced MARCKS phosphorylation and amplification of glucose-induced insulin release by carbamylcholine (25).

Protein kinase C activation elicits a variety of cellular responses by phos-

phorylating target proteins such as MARCKS on serine and threonine residues. The use of intact islet, permeabilized cells, islet homogenates, and subcellular fractions has helped identify several different protein substrates for protein kinase C in insulin-secreting cells. Among these substrates, a protein of M_r for 29 kDa was phosphorylated and may be associated with insulin secretory granules in insulinoma cells (22). Another protein kinase C substrate is a 37 kDa protein in rat islets that was phosphorylated by phorbol-12-myristate, 13-acetate treatment. A protein kinase C inhibitor (clomiphene) inhibited phorbol-12-myristate, 13-acetate-induced insulin release, and ^{32}P incorporation into this substrate (41). MARCKS has also been identified in rat pancreatic islets by immunoprecipitation (14). MARCKS is a calmodulin-binding protein, the phosphorylation of which results in rapid release of calmodulin, which can then activate calmodulin-dependent protein kinase, a component of the β-cell cytoskeleton. Activation of this kinase could phosphorylate components of the release system. Moreover, MARCKS is localized in cytoskeletal structures in its unphosphorylated state, and phosphorylation detaches it from the cytoskeleton. Thus carbachol-induced MARCKS phosphorylation might influence the calmodulin-dependent signal transduction pathways as well as the exocytosis events of insulin secretion.

Arachidonic Acid

Arachidonic acid is released from membrane phospholipids as the result of hydrolyzation by phospholipase A_2. Phospholipase A_2 in islet may be located in cellular membranes, and its activity requires Ca^{2+} (54). As is true for neutrophils, islet phospholipase A_2 activity may be regulated by a G protein. Investigators recently observed that carbachol stimulates arachidonic acid release from perifused islets (51) by stimulating phospholipase A_2.

Arachidonic acid is a long-chain unsaturated fatty acid containing 20 carbon atoms and 4 double bonds. In the resting state, most cellular arachidonic acid is esterified to membrane lipids, and the intracellular free concentration is very low. Under stimulating conditions, membrane phospholipids are hydrolyzed by phospholipase A_2, and arachidonic acid is released. Arachidonic acid is also released from DAG by DAG lipase. The increased intracellular free arachidonic acid may affect insulin release through several processes, including activation of protein kinase C and mobilization of intracellular Ca^{2+}. In islet, arachidonic acid–induced insulin release is inhibited by concentrations of trifluoperazine, which also inhibit protein kinase C activity. This stimulating effect is further reduced by prolonged pretreatment of islets with 12-*O*-tetradecanoylphorbol-3-acetate, which depletes protein kinase C activity of the islet (65). The importance of arachidonic acid in activating protein kinase C on insulin release was questioned recently. For example, Howell et al (5) demonstrated that 200 nM staurosporine caused a complete inhibition of arachido-

nic acid–induced phosphorylation without having any effect on arachidonic acid–induced insulin release.

Arachidonic acid may be able to amplify voltage-dependent Ca^{2+} entry when membrane depolarization is minimal. Patch-clamping studies with GH_3 cells indicate that arachidonic acid shifts the activation curve for high voltage–operated L-type Ca^{2+} channels to more negative potentials. In the presence of arachidonic acid, Ca^{2+} current flows through these channels at potentials only slightly above the resting potential, but in the absence of arachidonic acid, even at the same membrane potential, no Ca^{2+} current is present (103). Arachidonic acid induced a biphasic rise in $[Ca^{2+}]_i$ in Fura-2 loaded β-cells. The sustained phase of the rise was abolished by removal of extracellular Ca^{2+} and was amplified by depolarization with KCl, which indicates that arachidonic acid increases Ca^{2+} influx through Ca^{2+} channels (90). Arachidonic acid may also be able to mobilize intracellular Ca^{2+} from the endoplasmic reticulum of the β-cell (109).

Phosphatidic Acid

Membrane phospholipids are hydrolyzed by phospholipase D at the terminal phosphate diester bond, and phosphatidic acid is released as a product. Phosphatidic acid has been suggested as a possible mediator of insulin secretion. The following conclusions drawn from a number of studies support this proposal: (*a*) Exogenous phosphatidic acid increases insulin secretion (67); (*b*) exogenous phospholipase D is capable of stimulating insulin release from islets with a concurrent increase in intracellular phosphatidic acid levels (66); and (*c*) in statically incubated islets, 0.5 mM carbachol with 3 mM glucose present increased the phosphatidic acid level twofold (51, 52). More data are required to fully assess how phosphatidic acid might affect islet function.

MEMBRANE POTENTIAL, CYTOPLASMIC CALCIUM, AND INSULIN SECRETION

Electrical activity of the β-cell membrane plays a central role in stimulus-secretion coupling. When glucose concentration is below the level required to elicit insulin secretion, the β-cell is electrically silent. The resting potential is around –60 and –70 mV in 3 mM glucose. Raising the glucose concentration above 3 mM induces a slow depolarization of the β-cell. At glucose concentrations that elicit insulin release (> 7 mM), this depolarization is sufficient to bring the membrane to the threshold potential at which electrical activity is initiated. A characteristic pattern of electrical activity then ensues. In 10 mM glucose, this activity consists of slow oscillations in membrane potential between a depolarized plateau on which Ca^{2+}-dependent action potentials are superimposed and a more negative interburst interval. As glucose concentra-

tion is increased, the duration of the plateau increases, and the intervals between plateaus decrease. Continuous electrical activity finally occurs when the glucose level is above 20 mM. The resting membrane potential of the β-cell is governed by the activity of the K^+-ATP channel. Closure of this channel, either by glucose metabolism or by sulphonylureas, reduces the membrane K^+-permeability and leads to membrane depolarization. This event opens voltage-dependent Ca^{2+} channels and elicits β-cell electrical activity. The increased Ca^{2+} influx produces a rise in the intracellular calcium, which then stimulates insulin secretion.

K^+ Permeability, Membrane Potential, and its Regulation

The resting membrane potential of pancreatic β-cells is determined by the high K^+ permeability of the membrane. Several types of K^+-channels can be found on the membrane of β-cells: (*a*) The delayed rectifier K^+-channels are voltage dependent. The activity of this channel increases with depolarization. Its function is to repolarize the action potential. (*b*) K^+-Ca^{2+} channels are activated both by depolarization and by an increase in $[Ca^{2+}]_i$. Ca^{2+} influx during the action potential may lead to channel activation. These K^+-Ca^+ channels contribute to action potential repolarization but not to the slow waves. (*c*) ATP-sensitive K^+-channels are inhibited by high ATP concentrations. These K^+-ATP channels are the key link between K^+ permeability and glucose metabolism. They can be blocked by ATP in cell-free patches and by the metabolism of glucose in intact cells. It has been estimated that 10–25% of K^+-ATP channels are activated at resting conditions in mouse β-cells (3) vs 4% in rat β-cells (19). Using cell-attached patch recordings, investigators have established that 50% of the channels, which are active in the absence of glucose, are inhibited by 2 mM glucose, and more than 90% are inhibited at 5 mM glucose concentration (70). The inhibitory effect of ATP may not involve a phosphorylation event, since nonhydrolyzable ATP analogs are also effective. Thus ATP is thought to interact directly with a site on the channel or with an associated control protein. In intact cells, cytosolic ATP concentration at ~ 1 mM produced half maximal inhibition of K^+-ATP channel activity (73). However, most biochemical studies have failed to demonstrate the postulated changes of ATP in response to glucose stimulation (32, 78). Instead, glucose was found to decrease ADP levels and thus increase the ATP/ADP ratio in islets. It is believed that the ATP/ADP ratio determines channel activity in β-cells (24). However, this hypothesis has been challenged recently. Hopkins et al (39) observed that the absolute levels of adenine nucleotides (e.g. ADP and ATP) determine K^+-ATP channel activity rather than the ATP/ADP ratio. In the absence of ATP, ADP opened K^+-ATP channels at concentrations ranging from 10–500 μM with maximal activation between 50 and 100 μM.

It has been proposed that two kinds of ADP binding sites regulate the

K^+-ATP channel activity. One site specifically binds Mg-ADP and increases channel opening, whereas the other (the previously described ATP site) binds either ATP or ADP and decreases channel opening. To explore the influence of cytosolic-free ADP on the change of membrane potential induced by glucose, it is crucial to monitor the free ADP level in islets under basal and stimulated conditions. Relatively high analytical errors can occur in the individual measurement of free ADP in islet tissue because the creatine buffer used for this purpose is naturally of low abundance in the pancreatic islets. We recently developed a transgenic mouse that overexpresses rat brain creatine kinase in insulin-secreting pancreatic β-cells. The introduction of the creatine buffer system (created by culturing islets in 30 mM creatine) in combination with the high capacity of β-cell creatine kinase enabled us to determine the level of free ADP in perifused mouse islets. At baseline conditions with 2.5 mM glucose, the free ADP in these islets was 182 μM. When stimulated by 25 mM glucose combined with 30 mM K^+, the free ADP level dropped to 66 μM, and the ATP level in these islets was reduced from 2.93 to 1.93 mM. Insulin release underwent a 20-fold increase when stimulated by 25 mM glucose (Kesavan, Katsumata, Najafi, Santangel, Matschinsky, et al, unpublished data). These observations support the view that the alteration of free ADP in islet plays a decisive role in stimulation-secretion coupling.

A variety of pharmacological agents influence β-cell electrical activity as a consequence of their ability to activate or inhibit ion-channel activity. Of clinical importance are sulphonylureas (e.g. tolbutamide and glibenclamide). These compounds act as potent and specific blockers of K^+-ATP channel activity. Sulphonylureas initiate insulin release by direct interaction with a high-affinity binding site located on the plasma membrane of the β-cell. This binding site is thought to be present on the K^+-ATP channel or on a closely associated protein. Using photolabeling with radiolabeled sulphonylureas, investigators have found that the high-affinity sulphonylureas binding site is a polypeptide of ~ 140 kDa (1). Evidence indicates that ADP binds to and competitively displaces [^{3}H]glibenclamide from this high-affinity cell sulphonylureas-binding site of HIT cells (75). Because ADP cannot cross the plasma membrane, ADP and sulphonylureas have common binding sites on the outer surface of the HIT cell plasma membrane. Niki & Ashcroft (74) reported that ATP depletion reduced the [^{3}H]glibenclamide-binding activity of intact HIT cells, which suggests that phosphorylation of the glibenclamide-binding site in the pancreatic β-cells modulates its [^{3}H]glibenclamide-binding properties. Because the channel/receptor has not been characterized at the molecular level, whether the target for phosphorylation is the channel itself or is represented by another associated protein related to the sulphonylurea-binding activity remains unclear.

Role of Intracellular Calcium in Insulin Secretion

During the last two decades, we have gained much knowledge about how cytoplasmic Ca^{2+} participates in the insulin secretory process. The development of suitable indicators and the use of dual-wavelength fluorometry have enabled direct observation of how glucose and other secretagogues alter the $[Ca^{2+}]_i$ in individual pancreatic β-cells. Measurements with the fluorescent indicators quin-2 and fura-2 have provided support for the idea that stimulation of insulin release by different secretagogues is associated with a rise of $[Ca^{2+}]_i$.

SOURCES OF CA^{2+} FOR INSULIN SECRETION The increase of $[Ca^{2+}]_i$ induced by secretagogues is the result of enhanced influx Ca^{2+} across the β-cell membrane and release from intracellular Ca^{2+} storage sites (88). The first process is essential for the rise in $[Ca^{2+}]_i$ because (*a*) the extent of the rise in $[Ca^{2+}]_i$ is dependent on the concentration of extracellular Ca^{2+}, and (*b*) the blocking of L-type Ca^{2+} channels abolishes the rise in $[Ca^{2+}]_i$.

Two types of voltage-dependent single-Ca^{2+} channel currents with properties resembling those of L-type and T-type Ca^{2+} channels have been described in rat β-cells. The L-type channels are of particular significance for insulin secretion because they mediate the Ca^{2+} entry that initiates insulin release. The purified calcium channel contains five protein subunits: the 175 kDa α1 subunit, the 132/28 kDa α2δ disulphide-linked subunits, the 55 kDa β subunit, and the 32 kDa γ subunit (35). The primary structure of these subunits was determined by cloning their cDNAs. The structure of the α1 subunits forms the calcium-conducting pore. The α2δ and γ subunits are membrane-spanning glycated proteins, whereas the intracellularly oriented β subunit may be of cytoskeletal origin (15). L-type Ca^{2+} currents are activated by depolarization to potentials more positive than –60 mV. Ca^{2+} currents are maximal around –20 mV and are of sufficient magnitude to account for action potential depolarization. Inactivation of the currents probably involves two processes: Ca^{2+}-dependent inactivation due to preceding Ca^{2+} entry, and a slow, voltage-dependent inactivation with time constants in the range of seconds. The L-type Ca^{2+} current appears to underlie both action potential depolarization and the plateau potential, which implies that most of the Ca^{2+} influx required for insulin release flows through L-type Ca^{2+} channels. Dihydropyridine and phenylalkylamine bind with the α1 subunit of L-type Ca^{2+} channel and block insulin release.

The intracellular calcium storage compartment is the endoplasmic reticulum in pancreatic islet β-cells. Calcium is transported into intracellular storage sites by an ATP-dependent mechanism. Calcium storage within the endoplasmic reticulum is achieved by the calcium-coordinated protein matrix,

termed the reticulplasm. This protein contains the carboxy terminal endoplasmic reticulum retention sequence (Lys-Asp-Clu-Leu) and appears to bind calcium, as assessed by a calcium overlay-blotting technique. The endoplasmic retic-ulum has the capacity to bind a large number of calcium ions with low affinity. Evidence indicates that multiple calcium storage pools exist in pancreatic islets. IP_3 and ryanodine receptors are the two principal intracellular calcium channels responsible for mobilizing stored calcium. The IP_3 receptor contains typical membrane-spanning domains in the C-terminal region. These domains anchor the protein in the membrane with four of the subunits that combine to form the functional IP_3-sensitive calcium channel. The large N-terminal domain lies free in the cytoplasm, with the IP_3-binding site located at its end (69). Upon binding IP_3, the receptor undergoes a conformational change that may be related to the coupling process leading to channel opening (69). The IP_3 receptor can be phosphorylated stoichiometrically by cAMP-dependent protein kinase, protein kinase C, and Ca^{2+}-calmodulin-dependent protein kinase II. These phosphorylation sites potentially allow a complicated pattern of cross-talk mechanisms that regulate calcium release. The ryanodine receptor is another calcium-release channel. Ryanodine, caffeine, or calcium itself acts on this receptor to catalyze calcium release (9).

REGULATION OF INTRACELLULAR CALCIUM CONCENTRATION Alterations of $[Ca^{2+}]_i$ are induced either by change of L-type Ca^{2+} channels activity or by variation of intracellular calcium release. Ca^{2+} influx through L-type channels is influenced either by regulation of the β-cell membrane potential or by biochemical modulation of the Ca^{2+} channel itself. Glucose metabolism induces a change of ADP and of the ATP/ADP ratio in the cytoplasm, or sulphonylureas close the K^+-ATP channels and depolarize the β-cell, thereby activating voltage-dependent Ca^{2+} channels. Several intracellular second messengers induced by nonglucose secretagogues modify the activity of Ca^{2+} channels. One of the candidate messengers is cAMP. Activation of purified skeletal muscle L-type calcium channels is reportedly enhanced by phosphorylation of the α1 and β subunits by cAMP-dependent protein kinase (15). The fact that Ca^{2+}-channel activity declines in excised patches from pancreatic β-cells in the absence of Mg-ATP indicates that cAMP may also regulate the activity of Ca^{2+}-channel in β-cells (4). However, direct studies on β-cells produced different results, probably owing to unique recording conditions. L-type Ca^{2+} channels may also be regulated by neurotransmitters via a membrane-associated G-protein pathway.

Several mechanisms regulate the release of Ca^{2+} from intracellular storage. When pancreatic β-cells are stimulated by nonglucose secretagogues such as carbachol, phospholipase C is activated by G protein connected with the

receptor. Hydrolysis of membrane phospholipids increases the IP_3 level in islets. When IP_3 binds to its receptor, calcium contained in the IP_3-sensitive pools is released to the cytosol. This process has been studied in rat insulinoma cells and in islets from *ob/ob* mice (89, 76). The action of IP_3 on Ca^{2+} release has been observed either in membrane vesicles or in storage sites in their normal location within permeabilized neuronal cells. The response is extremely fast, reaching a maximum within 140 ms of adding IP_3 to synaptosomes (29). These studies revealed considerable variability in the sensitivity of IP_3-induced Ca^{2+} release, indicating that the IP_3-sensitive Ca^{2+} pools are not uniformly sensitive. Sensitivity of IP_3-induced calcium release may vary in two primary ways. First, IP_3 receptor sensitivity may change on the basis of the calcium content of the endoplasmic reticulum. Second, variations in sensitivity may depend on receptor heterogeneity arising from the presence of different gene products, from alternative splicing, or from posttranslation modification (9). In pancreatic β-cells, the currently held view of how IP_3 affects the β-cell handling of Ca^{2+} is that the IP_3 generated by activation of phospholipase C releases Ca^{2+} from the endoplasmic reticulum into the cytoplasm, from which it is then taken up by an IP_3-insensitive compartment. The stored calcium is then recycled to the IP_3-sensitive pool (37).

Using rat islet microsomes, Takasawa et al (98) found recently that Ca^{2+} stored in the IP_3-insensitive compartment was released by cyclic ADP-ribose (cADPR) at 1.0 μM. Cyclic ADPR, a low molecular weight metabolite of the pyridine nucleotide NAD^+, was discovered by Hon Cheung Lee and his colleagues in sea urchin eggs. It has been suggested that cADPR may function widely as a calcium mobilizing agent. The enzyme responsible for synthesizing cADPR (ADP-ribosyl cyclase) is present in many mammalian and invertebrate tissues. Two isoforms of this enzyme were recently purified from the ovotestis of the marine mollusk *Aplysia californica* and from dog brain. The *Aplysia* form is a 29 kDa and probably cytosolic enzyme, whereas the mammalian form is in excess of 100 kDa and is membrane associated. Using polymerase chain reaction (PCR), a mammalian homologue to the *Aplysia* cADPR cyclase cDNA was obtained from a human insulinoma, and the activity of the cDNA-derived protein converts NAD^+ to cADPR and nicotinamide (79). ATP increases the activity of this protein. Although no cADPR has been detected in islet tissue owing to the limited amount of sample, 1 μM of cADPR can induce Ca^{2+} release from islet microsomes. This cADPR-induced Ca^{2+} release was not blocked by heparin, which inhibits IP_3 binding to its receptor. Depletion of this cADPR-responsive Ca^{2+} pool by cADPR also decreased the effectiveness of ryanodine, which indicates that cADPR and ryanodine act at the same Ca^{2+} storage compartment (98). These data suggest that in pancreatic islet, cADPR functions as another second messenger of calcium mobilization.

An additional way to regulate the release of Ca^{2+} in pancreatic β-cells is Ca^{2+}-induced Ca^{2+} release (CICR). CICR, a property of the ryanodine receptor, was first described in muscle cells. Islam et al (44) have observed CICR in insulin-secreting RINm5F cells by using thimerosal, a sulphydryl reagent that opens the CICR channel. CICR may be important in the formation of Ca^{2+}-oscillation in pancreatic islet. These oscillations have been observed in individual pancreatic β-cells following glucose stimulation (36). Several types of cytoplasmic Ca^{2+} oscillations have been identified in individual pancreatic β-cells. Exposing mouse β-cells to 7–20 mM glucose alone induces a sinusoidal $[Ca^{2+}]_i$ oscillation with a frequency of 0.5–5/10 min. Cyclic AMP and carbachol induced different types of Ca^{2+} oscillation when glucose was present. The mechanism of this glucose-induced Ca^{2+} oscillation is unclear and may be caused by several factors. Corkey et al (57) reported that glucose stimulation induced oscillations of both ATP and O_2 consumption in pancreatic β-cells; these oscillations were similar to Ca^{2+} oscillation, which indicates that oscillation of glucose metabolism may control Ca^{2+}oscillations. The rhythm of Ca^{2+} oscillations seems to be an intrinsic property of β-cells rather than the result of neural pacemaker activity (108). This Ca^{2+} oscillation can improve the signal-to-noise ratio, particularly at low agonist concentration. The availability of both amplitude and frequency-encoded signaling enables Ca^{2+} to generate multiple signals in the cell. Ca^{2+} oscillation may therefore be a crucial component in stimulation-secretion coupling.

SUMMARY

Insulin release induced by nonglucose secretagogues is initiated from β-cell by a wide variety of stimuli through specific receptors or binding sites. Activation of receptors in turn generates or enhances the cytosol levels of cAMP, cADPR, IP_3, DAG, and AA. These second messengers then activate protein kinases, change the ion currents cross the cell membrane, and mobilize intracellular Ca^{2+}, thereby increasing phosphorylated proteins in the cytosol and augmenting $[Ca^{2+}]_i$. These events trigger exocytotic discharge of insulin. The crucial steps in receptor-mediated stimulation-secretion coupling and their relationship to glucose-stimulated insulin release is summarized in Figure 1. At the present stage of research, the general processes of secretagogue binding to receptors, of generating second messengers, of activating several types of protein kinase, and of altering the membrane potential as well as cytosol calcium levels has been intensively studied and qualitatively clarified. However, we know little about the exact nature of substrates of different protein kinases and their function in the insulin secretion process. With the help of

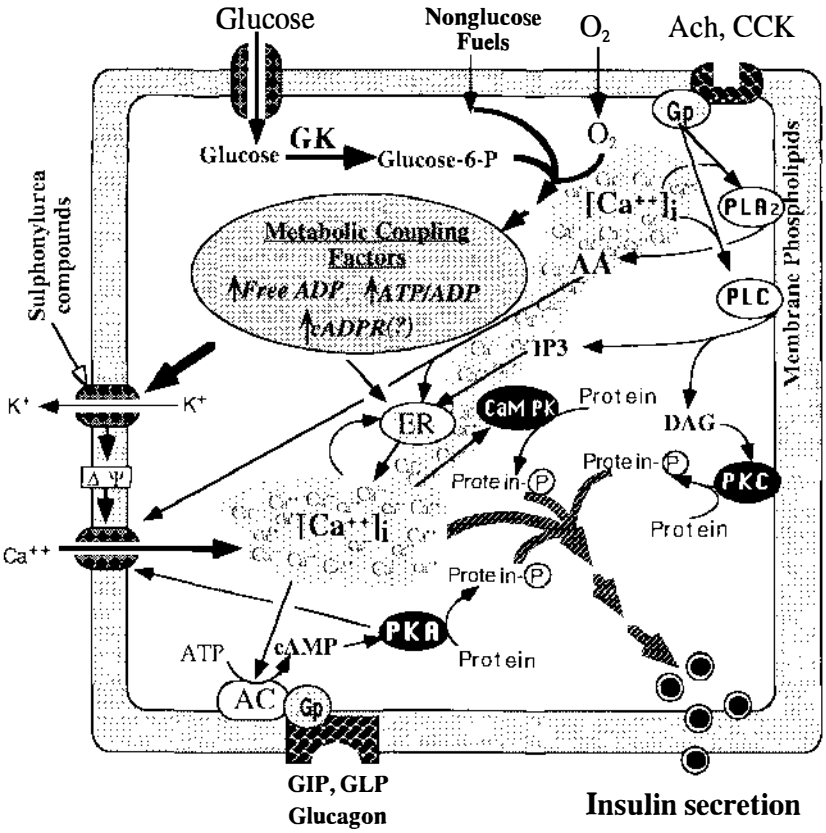

Figure 1 Schematic representation of selected receptor-activated steps in stimulus-secretion coupling and their relationship to selected processes of glucose-induced insulin release in pancreatic β-cells. Activation rather than inhibition and processes of glucose-induced insulin secretion as well as their modulation by different nonglucose secretagogues are emphasized. For further explanation see text. AA, arachidonic acid; AC, adenylyl cyclase; Ach, acetylcholine; cADPR, cADP-ribose; CaM K, calmodulin-dependent kinase; CCK, cholecystokinin; DAG, diacylglycerol; GK, glucokinase; GIP, gastric inhibitory polypeptide; GLP, glucagon-like peptide; Gp, G proteins; IP_3, inositol 1,4,5-triphosphate; PKA, protein kinase A; PKC, protein kinase C; PLA_2, phospholipase A_2; PLC, phospholipase C. Insulin synthesis stimulated by secretagogues is not considered.

molecular biology and protein chemistry, we expect that this gap will be filled in the near future.

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