Diabetologia (2003) 46:1297–1312
DOI 10.1007/s00125-003-1207-4

Diabetologia

Review

Fatty acid metabolism and insulin secretion in pancreatic beta cells

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Abstract

Increases in glucose or fatty acids affect metabolism via changes in long-chain acyl-CoA formation and chronically elevated fatty acids increase total cellular CoA. Understanding the response of pancreatic beta cells to increased amounts of fuel and the role that altered insulin secretion plays in the development and maintenance of obesity and Type 2 diabetes is important. Data indicate that the activated form of fatty acids acts as an effector molecule in stimulus-secretion coupling. Glucose increases cytosolic long-chain acyl-CoA because it increases the "switch" compound malonyl-CoA that blocks mitochondrial β-oxidation, thus implementing a shift from fatty acid to glucose oxidation. We present arguments in support of the following: (i) A source of fatty acid either exogenous or endogenous (derived by lipolysis of triglyceride) is necessary to support normal insulin secretion; (ii) a rapid increase of fatty acids potentiates glucose-stimulated secretion by increasing fatty acyl-CoA or complex lipid concentrations that act distally by modulating key enzymes such as protein kinase C or the exocytotic machinery; (iii) a chronic increase of fatty acids enhances basal secretion by the same mechanism, but promotes obesity and a diminished response to stimulatory glucose; (iv) agents which raise cAMP act as incretins, at least in part, by stimulating lipolysis via beta-cell hormone-sensitive lipase activation. Furthermore, increased triglyceride stores can give higher rates of lipolysis and thus influence both basal and stimulated insulin secretion. These points highlight the important roles of NEFA, LC-CoA, and their esterified derivatives in affecting insulin secretion in both normal and pathological states. [Diabetologia (2003) 46:1297–1312]

Keywords Insulin secretion, fatty acid, malonyl-CoA, long-chain acyl-CoA, incretin, hormone sensitive lipase, protein kinase C, exocytosis, acylation, metabolism.

Received: 14 April 2003 / Revised: 14 July 2003
Published online: 12 September 2003
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Abbreviations: ACS, acyl-CoA synthetase; ACC, acetyl-CoA carboxylase; BAT, brown adipose tissue; CPT, carnitine palmitoyl transferase; CL, citrate lyase; DAG, diacylglycerol; GSIS, glucose-stimulated insulin secretion; HSL, hormone-sensitive lipase; K_{ATP}, ATP-sensitive K^+ channel; LC-CoA, long chain acyl-CoA; PA, phosphatidate; PFK-1, phosphofructokinase-1; PKC, protein kinase C; PMA, phorbol myristate acetate; PC, pyruvate carboxylase; PS, phosphatidylserine; SNAP, soluble NSF-associated protein; SNAP-25, synaptosomal-associated protein of 25 kD; t-SNARE, target SNAP receptor; v-SNARE, vesicle SNAP receptor; VAMP, vesicle-associated membrane protein; VDCC, voltage-dependent Ca^{2+} channel; WAT, white adipose tissue.

Introduction

Type 2 diabetes, which accounts for 90 to 95% of diabetes worldwide, and obesity are prevalent and expensive diseases in Western society. Diabetes is estimated to affect 6% of the adult population and with a growth rate of 6% per annum, it is estimated that 200 to 300 million people will be afflicted by the end of the decade [1]. As obesity is the single most important risk factor for Type 2 diabetes, its rapid increase could well be driving this growth.

These diseases share two characteristics. First, insulin resistance is a characteristic of almost all people with Type 2 diabetes or obesity. In most cases of early diabetes and obesity, insulin concentrations are either normal or greatly increased. Obesity, with accompanying insulin resistance, is a powerful risk factor for

Type 2 diabetes. Furthermore, obesity and insulin resistance are found in people who are at an increased risk for developing these disorders, suggesting that they could be common pathological factors, or at least early events in their development. Second, obesity and Type 2 diabetes are often associated with hypertriglyceridaemia or increased circulating concentrations of NEFA [2, 3]. Therefore Type 2 diabetes can also be considered a lipid disorder as well as a disease of glucose tolerance [4] and it is possible that increased circulating lipid concentrations explain, at least in part, not only insulin resistance but also beta-cell dysfunction in Type 2 diabetes.

A role for the beta cell in Type 2 diabetes and obesity

Beta cells are involved in the abnormalities associated with both obesity and Type 2 diabetes. It has long been appreciated that obese humans and experimental animal models are hyperinsulinaemic and insulin resistant [5]. The development of obesity with the progression to Type 2 diabetes often involves a high-fat, high-sucrose diet. Under these conditions insulin secretion increases to accommodate the need to store glucose and excess fatty acids. The islet lesion in obesity is basal hypersecretion. In Type 2 diabetes, which can also involve basal hypersecretion, the main beta-cell lesion is a defect in the ability of glucose to incrementally stimulate insulin release. In addition, there is a reduction in the potentiating action of other substrates, such as amino acids or fatty acids, hormones or neuronal factors, in interacting with glucose to further stimulate secretion [6]. We propose that these lesions occur in susceptible people in whom the signal transduction enzymes, such as protein kinases, exhibit altered sensitivity to conditions that regulate long-chain acyl CoA (LC-CoA), the metabolically active form of long-chain fatty acids. Thus, obesity could be the initial condition which sets up the chain of events, with a subset of subjects in this pool developing Type 2 diabetes. If the beta cell lesion is prompted by both high fatty acids concentrations and high glucose, then an even smaller subset of lean subjects should develop Type 2 diabetes.

A role for adipocytes in Type 2 diabetes and the control of insulin release

The adipocyte is the main storage depot for triglycerides in the body. Stimulation of β1 and β3 adrenergic receptors found on white adipose tissue (WAT) and brown adipose tissue (BAT) activates hormone-sensitive lipase (HSL), causing lipolysis, the release of NEFA and a reduction of fat stores with an improvement of obesity-induced insulin resistance [7]. The β3-adrenergic receptor, which is predominately expressed on WAT and BAT, represents a potential anti-obesity target for drug treatment and for which several selective agonists have been developed [8, 9].

Short term treatment of rodents with such an agonist, CL 316,243, resulted in a twofold increase in energy expenditure as measured by O_2 consumption, a 50% reduction in food intake, and a rapid fourfold increase in serum NEFA that peaks within 5 min. Most striking, however, was a 50- to 100-fold increase in serum insulin concentrations that peak within 10 min [10, 11, 12]. Use of β3-receptor knockout mice showed that these effects are mediated exclusively by the expression of this receptor on fat cells. Re-expression of the β3-receptor in WAT completely restored the effect on serum NEFA concentrations and the dramatic rise in serum insulin concentrations [13]. In contrast, re-expression of the β3-receptor in BAT had the effect of only partially restoring the increase in O_2 consumption.

The fivefold increase in NEFA followed by a 50- to 100-fold increase in serum insulin shows that the hypothesized link from adipose to islet tissues is important. Given the rapidity and magnitude of this response an increase in hepatic glucose production or a reduction in insulin clearance can be ruled out. Thus, adipocyte products could play a major role in regulating insulin secretion in vivo. These products include, but might not be limited to NEFA or could depend on the physiological mix of NEFA contained in the adipocyte. Whether other products released from WAT with β3-receptor stimulation, in addition to NEFA, are acting directly on the beta cell remains to be determined.

Metabolism in beta cell stimulus-secretion coupling

High-energy intermediates. The molecular mechanism by which glucose and other fuels stimulate insulin release is still unclear. Fuel-induced secretion, like other regulated secretory processes, is dependent on extracellular free Ca^{2+}. However, beta cells possess a unique stimulus-response coupling system, which requires that the fuel stimulus be metabolized to initiate membrane electrical events, which lead to cell depolarization and secretion [14, 15]. Only fuels that stimulate insulin secretion stimulate change in electrical activity. Inhibition of fuel metabolism inhibits both secretion and electrical activity [14, 16]. Metabolism of glucose generates signals that modulate the activities of enzymes and ion channels, increasing the concentrations of intracellular messengers [17, 18]. These include high-energy intermediates and also adenine nucleotides [19, 20, 21], pyridine nucleotides [22, 23], and CoA derivatives [24, 25]. Among the latter is malonyl-CoA, an inhibitor of carnitine palmitoyl transferase (CPT)-1 that could cause a switch from

fatty acid oxidation to complex lipid formation. It should be noted that only fuels or fuel combinations that elevate malonyl-CoA stimulate secretion [25] and that inhibition of malonyl-CoA production inhibits secretion [26]. Glucose-stimulated insulin secretion (GSIS) is associated with inhibition of NEFA oxidation and increased lipid synthesis in pancreatic beta cells [24, 27, 28, 29]. Indeed, significant increases occur in the total mass of diacylglycerol (DAG) [30] and phosphatidate (PA) [18] in glucose-stimulated beta cells. Glucose and endogenous LC-CoA are the main sources of the glycerol and lipid components, respectively, of DAG and PA [28, 30]. In addition, exogenous fatty acids potentiate GSIS [25, 29, 31], possibly by providing additional acyl groups for LC-CoA formation or the synthesis of complex lipids.

Anaplerosis and cataplerosis. Accelerated acetyl-CoA production is undoubtedly essential for the rapid generation of reducing equivalents and ATP, and consequently for ATP-sensitive K^+ (K_{ATP}) channel closure. It is however not sufficient since substrates which are metabolized directly to acetyl-CoA, i.e. fatty acids and ketone bodies, are not secretagogues in the absence of glucose [32]. Thus, accelerated production of acetyl-CoA and oxidative events do not solely account for full induction of insulin secretion. However, anaplerosis is likely to be important in beta-cell activation for several reasons. Firstly, it is required for the efficient operation of either a pyruvate/malate or pyruvate/citrate shuttle allowing the production of cytosolic malonyl-CoA [32] and NADPH [33]. Approximately 40% of the glucose carbon entering the citric acid cycle is carboxylated in rat islets [34]. This is a very high percentage for a non-gluconeogenic tissue [35] and a cell synthesizing fatty acids at a low rate [36]. Secondly, the dose dependencies of anaplerosis, citrate, malate and malonyl-CoA accumulation in response to glucose correlate well with secretion in beta cells [37, 38]. Thirdly, methylsuccinate is a potent secretagogue in intact beta cells [39] and succinate directly promotes exocytotic release of insulin in permeabilized pancreatic beta cells [40]. Fourthly, phenylacetic acid reduces anaplerosis and insulin secretion in clonal beta cells (INS) and rat islets [37] by inhibition of pyruvate carboxylate.

Thus, glucose, glyceraldehyde and dihydroxyacetone, which feed directly into glycolysis are all secretagogues [14]. After their transformation to pyruvate they can be metabolized to both acetyl-CoA and oxaloacetate directly. However, glucose which provides anaplerosis is required for fatty acid- and ketone body-induced insulin release [41, 42]. Leucine (acetyl-CoA production) and glutamine (anaplerosis) synergize to promote secretion [42]. These features of beta-cell fuel stimuli favour the concept that acetyl-CoA production and anaplerosis are the earliest mitochondrial events synergizing to promote the production of coupling factors activating the beta-cell secretory process and is consistent with the idea that changes in lipid partitioning also play a key role in the regulation of insulin secretion [43, 44].

K_{ATP} channel-dependent and -independent actions of glucose

The consensus model of nutrient-stimulated secretion postulates that increased glycolysis and respiration due to glucose metabolism leads to accelerated ATP production and an increase in the ATP to ADP ratio [44]. This in turn closes the K_{ATP} channel, depolarizes the cell, increases the open time of voltage-dependent Ca^{2+} channels and raises intracellular Ca^{2+}. The increased Ca^{2+} then modulates kinases or other effector systems involved in secretion. The broad outlines of the K_{ATP} channel-dependent pathway described above are fairly well worked out as several components have been cloned [45, 46, 47]. However, this model does not prove entirely satisfactory given that K^+-induced secretion, which maximally increases Ca^{2+}, only transiently stimulates secretion [14]. In addition, recent reports have documented GSIS which is independent of the K_{ATP} channel [48, 49, 50]. This stimulation is dependent on glucose metabolism, has a normal concentration dependence, is shared by other nutrients, and is either independent of Ca^{2+} [50] or changes in Ca^{2+} [48, 51]. In addition, the action of glucose in the absence of Ca^{2+} can be mimicked by long-chain fatty acids provided that activators of protein kinase C and protein kinase A are present [52]. This is consistent with the notion that both glucose and NEFA are signalling through cytosolic LC-CoA.

A more inclusive model of nutrient-stimulated secretion involves two arms of signal transduction which occur simultaneously (Fig. 1). One arm is de-

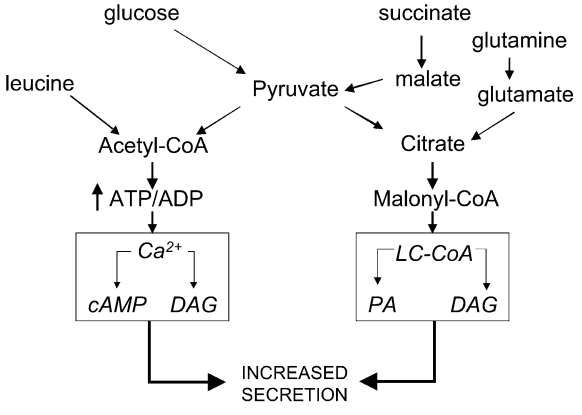

Fig. 1. Proposed dual signalling pathways involved in the physiological stimulation of insulin secretion by nutrients. The primary (Ca^{2+} and LC-CoA) and secondary (cAMP, DAG and PA) intracellular messengers generated in each pathway are shown boxed

pendent upon modulation of the K_{ATP} channel evoked by changes in the ATP:ADP ratio due to glycolysis and the accelerated production of acetyl-CoA. Secretory input from this arm would be an increase in cytosolic Ca^{2+} and secondary to this, changes in cAMP and products of phospholipase activation such as DAG. The other arm is dependent upon anaplerotic input into the TCA cycle, generation of excess citrate with its accumulation in the cytosol, and increases in cytosolic malonyl-CoA [44, 53]. Secretory input from this arm would be increased cytosolic malonyl-CoA and LC-CoA, and secondary to this, the increased synthesis of complex lipids such as PA and DAG. This is consistent with the ability of hydroxycitrate to prevent the rise in cytosolic LC-CoA by blocking malonyl-CoA production and thus inhibiting nutrient-stimulated insulin secretion [26]. This inhibition can be overcome by the addition of exogenous NEFA. Complementing this result is the finding that expression of a dominant negative form of acetyl CoA carboxylase (ACC), the key enzyme in the synthesis of malonyl-CoA, prevents its rise and also inhibits secretion [54]. Thus, the model predicts that signalling through both arms would be required for a normal secretory response to nutrients. Other nutrient secretagogues also fit this model. Methylsuccinate which is converted to succinate in the cytosol enters the mitochondria in exchange for malate. In turn, malate is converted to pyruvate in the cytosol by malic enzyme which then follows the same metabolic route as pyruvate derived from glucose (Fig. 1).

Oscillations in insulin secretion

Insulin secretion is normally oscillatory in vivo, in isolated islets and clonal beta cells [55]. The physiological importance of insulin oscillations for beta-cell function is suggested by their loss or impairment in Type 2 diabetic patients and their near relatives [56, 57]. Since oscillations increase the potency of insulin on target tissues, the loss of oscillations could contribute to an insufficient pattern of insulin production that leads to diabetes. Metabolites that have been measured at sufficiently frequent intervals also oscillate in beta cells, such as the ATP:ADP ratio [58, 59]. We have suggested that this pattern could be crucial for generating the wide swings in signals required for metabolism-secretion coupling. Such high and low values would also help to explain how both the ATP:ADP ratio and O_2 consumption show average increases in stimulated beta cells, despite the well established observation that an increase in the ATP:ADP ratio inhibits O_2 consumption [60]. Oscillations would also prevent the continuous increase of stimulatory metabolites which would probably lead to desensitization and down-regulation of responses. Our concept of the mechanism of the metabolic oscillations is based on detailed studies of spontaneous oscillatory glycolysis in skeletal muscle extracts [55]. These oscillations are driven by autocatalytic activation of the muscle isoform of the key glycolytic enzyme phosphofructokinase-1 (PFK-1) by its product fructose 1,6-bisphosphate resulting in large oscillations in the ATP:ADP ratio. The muscle isoform of this enzyme, PFK-M, has recently been shown to be the dominant PFK activity in beta cells [61].

Our previous work has established that the time course of changes in metabolic and ion parameters such as changes in the ATP:ADP ratio or in metabolites such as malonyl-CoA occur before increases in Ca^{2+} or insulin secretion [58, 62]. This might allow for complex regulation of various targets dependent on the phasing of such oscillations. For example, it could be important for the peak of the ATP:ADP ratio to coincide with the trough of the LC-CoA concentration since these two metabolites have opposite effects on the K_{ATP} channel [63, 64, 65, 66].

NEFA metabolism in the beta cell

Fatty acids, not glucose, are believed to be the major endogenous energy source for unstimulated islets [67]. This is consistent with the observations that islets maintain high rates of oxygen consumption in the absence of exogenous fuels but contain little glycogen [68]. Stimulation of islets by glucose diminishes fatty acid oxidation and increases total respiration [27, 28, 29]. Thus, glucose stimulation seems to shift the beta cell from fatty acids to glucose as an oxidative fuel. This occurs through glucose conversion to malonyl-CoA, which inhibits CPT-1 and thus blocks LC-CoA oxidation by preventing transport into the mitochondria [32]. Glucose causes marked alterations in the acyl-CoA profile of clonal pancreatic beta cells, with the largest (fivefold) and earliest (by 2 min) change occurring in malonyl-CoA [24, 25]. There is a tighter correlation between secretion and LC-CoA concentrations than between secretion and malonyl-CoA concentrations. This observation coupled with the fact that de novo fatty-acid synthesis is very low in the beta cell [27] indicates that malonyl-CoA is used as a "switch" compound not as a precursor or effector molecule like LC-CoA. Inhibition of mitochondrial NEFA (LC-CoA) oxidation increases LC-CoA in the cytosol and could explain the observed increases in de novo synthesis of DAG and phospholipids after stimulation. The increases in LC-CoA, PA and DAG resulting from glucose stimulation could directly activate protein kinase C (PKC) isoforms [31] or modify the acylation state of key proteins involved in regulating ion-channel activity and exocytosis [69].

Multiple steps are involved in controlling cytosolic LC-CoA concentrations (Fig. 2). Long-chain NEFA

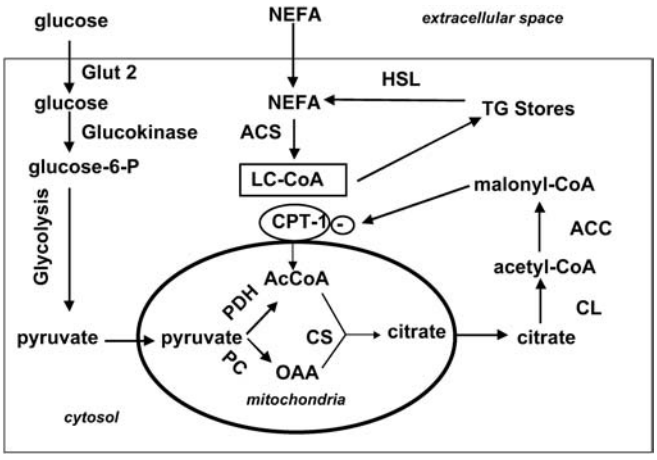

Fig. 2. Fatty acid metabolism in the pancreatic beta cell. Pathways for the formation of cytosolic LC-CoA stemming from the metabolism of glucose and either exogenous or endogenously generated free fatty acids are illustrated. *HSL* hormone sensitive lipase, *TG* triglyceride, *LC-CoA* long-chain acyl-CoA, *ACS* acyl-CoA synthetase, *PC* pyruvate carboxylate, *PDH* pyruvate dehydrogenase, *CS* citrate synthese, *CL* citrate lyase, *ACC* acetyl-CoA carboxylase

seems to be exclusively transported into the cell by free diffusion with no obvious requirement for active transport [70]. The very high avidity with which NEFA partition into phospholipid membranes makes it almost impossible for transport proteins to compete with the very rapid physical "flip-flop" that has been documented. The model predicts that even at NEFA concentration as low as 2 nmol/l in the aqueous phase, the concentration in the bilayer is 2 mmol/l or 2 mol% relative to phospholipids, thus allowing for large transmembrane fluxes. For these reasons, extracellular NEFA can be expected to rapidly distribute in the available lipid bilayers and to move rapidly from cell to cell, potentially acting as paracrine mediators. In addition, islets express low-density lipoprotein receptors [71] and lipoprotein lipase [72] and could also obtain fatty acids from circulating lipoproteins.

The first step in the control of NEFA partitioning is substrate supply. In the resting state, when glucose is low, fatty acids are converted to LC-CoA by acyl-CoA synthetase (ACS) and enter the mitochondria where they are oxidized via the β-oxidation pathway for the energy production needed [28]. The shift from NEFA to glucose as an oxidative fuel occurs through glucose conversion to the "switch" compound malonyl-CoA. This compound in turn inhibits CPT-1, found on the outer mitochondrial membrane, and thus blocks LC-CoA entry into the mitochondria [73]. Cytosolic concentrations of LC-CoA esters are controlled by feedback inhibition of ACS and are buffered by fatty acid and LC-CoA-binding proteins [74]. The total CoA pool is fixed over short periods of time and distributed unevenly between cytosolic and mitochondrial pools which are not interchangeable [75]. Thus, the maximal LC-CoA concentration is limited by the total CoA pool and by the compartmental distribution. There is a reciprocal relationship between these two pools, such that when transport into the mitochondria is limited, the concentration in the cytosol should increase. In response to CPT-1 inhibition, measured changes in total cellular LC-CoA can increase or decease depending on the percentage of the total made up by the mitochondria, which tend to contain the higher concentration. However, short term increases in extracellular NEFA might increase LC-CoA in both compartments. In addition, increased serum NEFA or certain drugs or steroids can increase the total CoA pool over the course of hours to days [76]. The cytosolic free concentration of LC-CoA is not known in any cell, but the total concentration has been calculated to be 95 and 220 nmol/g dry weight in livers of fed and fasted rats, respectively [76]. Based on Scatchard analysis of palmitoyl-CoA binding in permeabilized beta cells, the half-maximal cytosolic concentration in beta cells is estimated to be about 1 μmol/l, suggesting that this is the resting free concentration and the balance is bound to proteins or membranes [77].

Potentiation of GSIS by extracellular NEFA

The rapid effect of NEFA to potentiate GSIS in vitro, while having little effect on secretion at non-stimulatory glucose concentrations, would suggest that they act as incretins [25, 31, 78, 79, 80]. This effect has also been documented in vivo, where NEFA increase serum insulin concentrations in animals [81] and humans [82]. Under normal physiologic conditions the necessity for simulatory glucose can stem from the additional need for one or more of the following signals generated by glucose: (i) an increased influx of Ca^{2+}, (ii) an increased ATP:ADP ratio acting distal to the K_{ATP} channel, (iii) increased production of α-glycerol phosphate as a precursor to complex lipids, or (iv) increased malonyl-CoA required for inhibition of CPT-1. The observations that palmitate is both oxidized [28, 83] and also results in increases in cytosolic Ca^{2+} [78, 84] suggest glucose-like actions in stimulating secretion. Another possibility is that the LC-CoA derivative of the fatty acid directly modulates multiple effector systems in stimulating secretion [31, 63, 69, 77, 85] or that its esterification into complex lipids provides the necessary signalling factors in potentiating secretion [29, 78, 83, 86, 87, 88]. Several descriptive studies dealing with extracellular NEFA have shown that an increase in chain length, peaking around C16, and increasing saturation correlates with an increase in insulinotropic action of NEFA [80, 89, 90].

The requirement for circulating NEFA in maintaining the response of the pancreas after a fast highlights the physiological significance of this interaction [90, 91, 92]. These studies extended our understanding of

chain length and saturation in modulating the potentiation of glucose as well as suggesting that the specific mixture of NEFA is also important. It was hypothesized that for a given concentration of glucose the response of the pancreas is regulated not only by the concentration of NEFA, but also by the composition of this pool. In more recent work these findings included not only GSIS but also insulin secretion stimulated by amino acids and KCl-induced depolarization, suggesting that a critically important fatty acid-dependent step exists late in the stimulus-secretion coupling pathway [86]. The nature of this lipid-derived factor, its site or mechanism of action are not known. Interestingly, work in a HSL knockout mouse shows a requirement for lipolysis in nutrient-stimulated insulin secretion [93]. Findings showing that palmitate and myristate can substitute for glucose in augmenting the Ca^{2+}-independent pathway of secretion in islets [52] and could involve protein acylation [94] are consistent with this conclusion.

The beta cell response to hyperlipidaemia

NEFA have very different effects on insulin secretion depending on the specific NEFA and the length of time of exposure [95]. Short-term exposure of islets or beta cells to saturated long-chain NEFA provide a powerful potentiation of GSIS, whereas long-term exposure results in increased basal secretion and a blunted response to glucose. This dichotomy in response has been shown both in vitro [26, 89] and in vivo [81, 82, 96]. At early time points after lipid infusion (<6 h), glucose-stimulated secretion in these studies is enhanced, whereas measurements at 24 and 48 h show mostly an inhibition. Studies in vivo using heparin and lipid infusion do not always show inhibition at longer time points. A recent study [97] shows that long-term exposure to saturated NEFA results in enhanced GSIS, with the development of insulin resistance. However, unsaturated NEFA exposure results in impaired GSIS without insulin resistance. Epidemiological evidence links the ingestion of saturated NEFA with hyperinsulinaemia and insulin resistance [98].

These opposite effects of NEFA can be explained by: (i) desensitization of pathways due to continuous stimulation; (ii) the presence in the cell of enzymes with different concentration dependencies for regulation by LC-CoA, that are either stimulated, e.g., PKC [31], or inhibited e.g., PKC [31, 99], the adenine nucleotide translocase [100] and glucokinase [101]; and (iii) the ability of NEFA or LC-CoA to alter gene expression [102, 103]. Therefore, it is predicted that the response to NEFA or the resulting increase in cytosolic LC-CoA will depend on the concentration achieved and the length of time of the exposure. Accordingly, it can be hypothesized that hyperlipidaemia and increased NEFA are causally implicated in the progressive alteration in glucose metabolism in pancreatic beta cells and could explain the glucose recognition defect of islet tissue in subjects with Type 2 diabetes. The effect seen in isolated islets with chronic fatty-acid treatment causing a left shift in the glucose dose response curve, basal hyper-secretion and diminished stimulation by glucose, could involve increased activity of hexokinase, due to a rise in maximal activity and deinhibition by lowered glucose 6-phosphate concentrations [104]. The drop in glucose 6-phosphate in turn is most likely a result of increased PFK activity, caused by a rise in maximal activity and decreased concentrations of the inhibitor citrate because of decreased citrate synthase activity [104]. Abnormal sensitivity to glucose also results from chronic exposure to increased concentrations of glucose [53]. Decreased insulin secretion in response to glucose is preceded by enhanced sensitivity to glucose in the partially pancreatectomized model; this could be due to an increase of LC-CoA and products formed from LC-CoA, caused by glucose through the production of malonyl-CoA [24]. Thus, both hyperlipidaemia and hyperglycaemia probably cause an increase in cytosolic LC-CoA but by different mechanisms.

Chronic exposure of the beta cell to increased concentrations of NEFA inhibits insulin secretion [102] and biosynthesis [106], the expression of the beta cell transcription factor PDX-1 [107], the Glut-2 glucose transporter [107], ACC [102] while increasing CPT-I expression [103]. Thus, beta cell "glucolipoxia" probably plays an important role in the causes of obesity-associated Type 2 diabetes [32]. Within the framework of this hypothesis, alterations in malonyl-CoA production and in the expression of enzymes controlling lipid partitioning (ACC, CPT-I, HSL, and others) play important roles.

Hormone-sensitive lipase and lipid signalling in the beta cell and adipose tissue

Another source of NEFA available to raise beta cell cytosolic LC-CoA is its own triglyceride stores. Hormone-sensitive lipase (HSL) is a unique fatty acyl hydrolase expressed in the key tissues of insulin production and action: the pancreatic beta cell [108, 109, 110], the adipocyte [111] and skeletal muscle [111]. New evidence suggests that HSL, the rate-limiting step in triglyceride hydrolysis, plays a pivotal role in energy homeostasis via its roles in fat cells and beta cells [93, 112]. Consistent with this finding, HSL-knockout mice show reduced GSIS both in vivo and in isolated islets. These data provide important evidence that NEFA, their CoA derivatives or complex lipids formed from them are critical coupling factors in nutrient signalling in the beta cell. Recent evidence indicates that lipolysis of beta-cell triglyceride stores could play a central role in the action of incretins such

as GLP-1 in potentiating GSIS via increased intracellular cAMP [113, 114].

HSL transcripts have been studied in fat, testicle and beta cells [111]. The regulation of HSL transcription has mainly been studied in fat, where it is increased with fasting and diabetes [111]. Increased glucose induces the HSL gene in the beta cell with a resulting twofold increase in HSL protein and enzymatic activity [110]. This is associated with high basal secretion [53] in accordance with the view that lipid signalling molecules are implicated in glucose-stimulated secretion [110, 113]. Although HSL transcription is controlled, most of the regulation of its activity in adipocytes seems to be post-translational. β-Adrenergic stimulation of adipocytes results in the PKA-mediated phosphorylation of HSL and perilipin, the protein that coats the lipid droplets in the basal state. Following this phosphorylation, HSL translocates from the cytoplasm to the surface of the lipid droplet, while perilipin shifts in the opposite direction [111]. The relative importance of HSL and perilipin in this process is not completely established but both appear to be essential.

HSL is a fatty acyl hydrolase that is promiscuous in its range of substrates, cleaving DAG, triglycerides and to a minor degree, MG, as well as fatty acyl esters of cholesterol, steroid hormones and retinoic acid [111]. The greater activity against DAG than triglyceride is notable and could explain the marked increase of DAG in several tissues of HSL-deficient mice [115]. However, the great number of substrates and products of HSL implies that a number of compounds and pathways must be considered as mediators for the pathogenic mechanisms of HSL deficiency.

Potential mediators and targets of LC-CoA esters

LC-CoA or products derived from them, such as complex lipids, are potent regulators of enzymes, ion channels and various signal transducing effectors in many cell types (Table 1). These targets include the adenine nucleotide translocase, CPT-1, the tricarboxylic acid carrier, the nuclear thyroid hormone receptor, K^+_{ATP} channel, and several ATPases [116]. Of particular interest in this context, LC-CoA esters modulate the activity of proteins that contain adenine or guanine nucleotide binding sites, possibly as a consequence of the similarities in structure with coenzyme A [32]. NEFA, possibly via LC-CoA and/or complex lipid production are essential for insulin secretion in response to both fuel and non-fuel stimuli [86]. For instance, lysophosphatidate and PA might act as beta-cell signalling molecules. Lysophosphatidate, which potently promotes insulin release in rat islets [117] is the first phospholipid synthesized from LC-CoA, the precursor of many signalling phospholipids and an established signalling molecule [118, 119].

Table 1. Potential mediators and targets of LC-CoA

LC-CoA targets	Acylation targets
PKC	SNAP-25
Ca^{2+} ATPase	VAMP
Adenine Nucleotide Transferase	Synaptotagmin
CPT-1	CSP
HSL	VDCC (β-subunit)
K^+-ATP Channel	PKC

Beta-cell proteins and effector systems affected by LC-CoA either through their binding or acylation are listed. PKC, protein kinase C; HSL, hormone-sensitive lipase; CSP, cysteine string protein

Five non-exclusive potential targets could be involved in the modulation of GSIS by fatty acids: (i) LC-CoA, either directly or indirectly via DAG production could activate C-kinase enzymes [31, 43]; (ii) a rise in cytosolic LC-CoA could directly cause the exocytotic release of insulin [69]; (iii) LC-CoA could modulate K_{ATP} channel activity directly or via complex lipid formation. Thus, various lysophospholipids [120] and phosphoinositide [121] are potent inhibitors of beta cell K_{ATP} channels whereas LC-CoA increases the open-state probability of K_{ATP} channels in the beta cell [63, 64, 65, 66]. (iv) Stimulation of Ca^{2+}-ATPases by increases in cytosolic LC-CoA has been observed in clonal beta cells [77]. (v) Inhibition of HSL or other lipase activity is observed with increases in cytosolic LC-CoA [116, 122].

Of these targets, the action of LC-CoA on two would be predicted to be a net positive for insulin secretion: stimulation of various PKC isoforms, and direct stimulation of exocytosis. These positive actions seem to be the dominant effects, as indicated by the immediate stimulatory effect of exogenous NEFA on glucose-stimulated insulin secretion. In contrast, activation of either K_{ATP} channels or Ca^{2+}-ATPases would be expected to inhibit secretion by lowering cytosolic Ca^{2+} by different mechanisms. However, it is not clear whether the negative-feedback inhibition of HSL in either the beta cell or the adipocyte after a rise in serum NEFA concentrations would affect secretion. These latter negative effects might simply be quantitatively unimportant or less important under physiological conditions. Another intriguing possibility is that in the context of oscillations in glycolysis, intracellular Ca^{2+} and secretion [59, 114, 123, 124, 125, 126], they may actually contribute to the recovery of resting cytosolic Ca^{2+} after its increase, if LC-CoA oscillates out of phase with the ATP:ADP ratio. This scenario would be distinct from the incretin effects of exogenous NEFA which might be more "global" and long-lived.

Calcium influx and stimulation of exocytosis

In excitable cells, such as beta cells, voltage-dependent Ca^{2+} channels (VDCC) are the predominant gate-

keepers for cellular Ca^{2+} influx important in many vital cellular processes, including insulin secretion [17]. These channels are hetero-oligomeric protein complexes composed of at least four subunits, α1, β, α2, δ [127]. There is ample evidence that L-type VDCC play a pivotal role in beta-cell secretion, although the precise subunit composition of the channels involved and their metabolic modulation are not known [17, 128]. This goal has been complicated by the fact that all types of VDCC, including the two L-type classes α1c and α1d and four different β subunits are expressed in pancreatic beta cells [46, 47, 129].

The regulated release of insulin from the beta cell seems to utilize a mechanism that is highly conserved across many cell types including neuroendocrine cells [130]. Exocytosis is a sequential and multi-step process involving margination of granules, their docking at the plasma membrane, possible priming mechanisms, membrane fusion and the dissociation and recycling of exocytotic components. The consensus model is termed the soluble NSF-associated protein receptor or SNARE hypothesis where a vesicle-bound protein (v-SNARE) associates with a protein on a target membrane (t-SNARE) forming a complex which brings the secretory vesicle into extremely close approximation with the plasma membrane [130]. The t-SNARE proteins are syntaxin (four isoforms) and synaptosomal-associated protein of 25 kD (SNAP-25) (three isoforms), while vesicle-associated membrane protein (VAMP) (three isoforms) is the vesicle-bound protein. The Ca^{2+} sensitivity of exocytosis is thought to be mediated by another vesicle-associated protein, synaptotagmin, which normally acts as a brake on membrane fusion.

Secretory granules can be divided into those which correspond to a readily releasable pool, which may include both primed and unprimed vesicles, and to a separate reserve pool [131]. The latter granules require mobilization, docking and a "priming" event which could utilize ATP while the former are release competent. Recently, the ready releasable pool has been quantitated by immunologic detection of the docking complex and shown to be responsible for KCl-induced insulin release as well as the first phase of GSIS [132]. Inhibition of GSIS by hexammineco-balt (III) chloride, which was found to act by stabilizing the docking complex, is consistent with this conclusion [133].

Modulation of protein kinase C isoforms in the beta cell

There is no doubt that exogenous NEFA increases LC-CoA and potentiates GSIS and that this action is physiologically relevant [25, 86, 92]. Therefore, what is missing is a signalling cascade linking changes in LC-CoA to changes in insulin release. PKC isoforms are reasonable candidates for this cascade to either initiate secretion or augment GSIS as they respond to both lipid signals and Ca^{2+} [31, 134]. An alternative model could involve modifications of proteins not involving phosphorylation but rather acylation, or a combination of both processes possibly at different points in signal transduction after the generation of LC-CoA [94, 135].

Table 2. Differential regulation of PKC isoform classes

	CO-FACTORS			LC-CoA
	PS	DAG	Ca^{2+}	
Conventional PKC (cPKC)	X	X	X	+
Novel PKC (nPKC)	X	X		no action/–
Atypical PKC (aPKC)	X			++

To date there are 12 known PKC isoforms, of which 7 are expressed in the pancreatic beta cell: cPKC (α, βII); nPKC (δ, ε, μ); aPKC (ι, ζ). The isoforms are classified based on the linear structure of and co-factor binding to their regulatory domains. The catalytic domains are highly conserved across all isoforms

PKC is a family of 11 or 12 isozymes depending on nomenclature, which are divided into three classes, based on structure and co-factor requirements (Table 2). The conventional class (cPKC) requires phosphatidylserine (PS), DAG and Ca^{2+}, while the novel class (nPKC) does not require Ca^{2+}. The atypical class (aPKC) has only a known requirement for an acidic phospholipid such as PS and therefore little is known about its regulation. PKC-μ can be considered a separate class of kinase as PKD or a nPKC isoform having a modified phorbol ester binding site (C1 domain) and a putative transmembrane leader sequence. Unlike many enzymes, cPKC and nPKC isozymes require intracellular translocation and targeting to membrane surfaces for their activation [136]. The mechanism of this targeting involves both lipid (C1) and Ca^{2+} binding domains (C2) as well as protein–protein interactions with adaptor molecules contained in the cytosol [136]. The beta cell expresses seven isoforms, PKC-α, βII, δ, ε, ι and ζ as well as PKC-μ [31, 137, 138]. Although the beta cell contains isoforms of most major protein-kinase families; the regulation of the isoforms within a family is similar, except for PKC. This implies that in the case of other kinases, isozymes could provide redundancy for critical processes, whereas in the case of PKC these isoforms could underpin different cellular functions. Thus, it is of considerable interest to determine the physiological determinants of activation for the different PKC isoforms.

The stimulus-secretion coupling of some non-nutrient secretagogues occurs via PKC in receptor-mediated events linked to phospholipase C [139]. Phospholipase-C activation generates DAG, which translocates and activates PKC isoforms to phosphorylate endogenous substrates. Down-regulation of PKC isoforms by

chronic activation and the use of inhibitors suggests that PKC-α, βI / βII and ε mediate such pathways [140]. In contrast, the role of PKC in glucose-induced insulin secretion is unresolved, with several arguments for and against its involvement [141]. Glucose causes a rise in DAG [24, 30, 142] and promotes the translocation of PKC-α in the beta cell [143, 144, 145]. This is consistent with the observation that the mass of PKC-α correlates with the ability of phorbol myristate acetate (PMA), a high affinity surrogate for DAG, to stimulate secretion [146]. Therefore, the short-term activation of PKC is thought to be a positive signal for insulin secretion as seen by the effects of either phorbol esters or cell permeant diacylglycerols.

We have recently shown that down-regulation of PKC activity by chronic phorbol ester stimulation led to the differential loss of PKC isoforms as reflected in the loss of Ca^{2+}-dependent PKC activity seen in HIT cells [31]. Overnight exposure to 200 nmol/l PMA resulted in the down-regulation of PKC-α, βII, and ε with the masses of PKC-δ, ι, ζ unaltered. Whereas PKC-μ was not down-regulated, its mass was enriched in a Triton-X100 soluble membrane fraction [31]. After down-regulation, GSIS was not only preserved but enhanced, while the absolute potentiation due to exogenous NEFA did not change [31]. Therefore, the incremental potentiation due to NEFA was reduced by 35%, consistent with the loss of specific PKC isoforms. In line with this result is a report showing the blocking of NEFA-stimulated secretion by inhibitors of cPKC and nPKC isoforms in perifused rat islets [147]. In addition, we have shown that KCl-induced insulin secretion was also enhanced after PMA down-regulation [140, 146]). This would suggest that an increased sensitivity of exocytosis to Ca^{2+} and/or an increase in the ready releasable pool of secretory granules was due to an increased vesicle priming or to the prior inhibition of exocytosis. Recent work has shown that the enhanced secretory responses, both basal and stimulated, after PMA-induced down-regulation requires the presence of the phorbol ester in the cell [146]. This implies that DAG could also couple to secretion via either PKC-δ or μ; however, the data do not exclude involvement of a non-PKC target.

LC-CoA and PA can modulate the activity of different classes of PKC and their interaction with DAG and PS. PA has been shown to strongly augment the stimulation of PKC-ζ by PS [148]. Phosphatidylserine alone caused a slight stimulation above background (6%±2, $p<0.01$) while the combination of PS and PA caused a sixfold increase above the activity seen with PS alone. In addition, several long-chain acyl-CoA esters in combination with PS also stimulated cPKC activity fourfold and aPKC activity eightfold above PS alone [31]. Short-chain acyl-CoA esters were without effect in the presence of PS or PS plus DAG.

Previous studies have documented effects of PA [148] or PI-3,4,5-trisphosphate [149], in activating PKC-ζ. Recent evidence shows that the lipid-dependent kinase, PDK-1, activates PKC-ζ in a PI-3,4,5 P_3-dependent fashion by phosphorylation of threonine-410 of its activation loop [150]. Thus, a possibility still to be explored is that new regulators of islet PKC play a role in the potentiation of GSIS by NEFA.

Lipid-dependent translocation of PKC

A study in islets examining the effect of palmitate on beta-cell physiology showed that it did not inhibit the K_{ATP}-channel, or alter the membrane potential or the ATP:ADP ratio but modestly increased intracellular Ca^{2+} [78]. This rise in intracellular Ca^{2+} only increased secretion when glucose was increased, in keeping with action of the fatty acid as an incretin. A follow-up study showed that palmitate did not increase phospholipid turnover but did translocate PKC activity to a membrane fraction, only in the presence of stimulating glucose [43]. Significantly, blocking the metabolism of this NEFA (activation to LC-CoA) also blocked its ability to translocate PKC activity and stimulate insulin secretion. This suggests that either the LC-CoA or its esterification into a complex lipid such as DAG or PA was required for this effect.

Translocation of both PKC-α and ζ has been directly shown in response to glucose or glucose plus NEFA in islets and clonal beta cells [31, 144]. In the presence of glucose, exogenous oleate rapidly (3 min) enriched a total particulate fraction with PKC-ζ, consistent with its involvement in potentiated secretion [31]. NEFA addition to many cell types, including platelets, hepatocytes and myocytes, have shown PKC isoform translocation [151]. A recent study has shown through confocal microscopy that different NEFA translocated fluorescent-tagged PKC-γ and ε in COS-7 and CHO-K1 cells [152]. The targeting was rapid and reversible and varied depending on the NEFA species used. The activity of cPKC has been enhanced by palmitoylation through increased targeting to cell membranes [135]. Of interest, PKC isoform translocation was much slower and not reversible when NEFA were combined with cell permeant diacylglycerols. In addition, this group found that each PKC isoform had a unique translocation pattern that depended on the stimulus involved, suggesting that this mechanism confers specificity to the cellular response.

Modulation of secretory granule transport and exocytosis

Both protein kinases C and A seem to modulate granule pools in neurons and various neuroendocrine cells including beta cells, but perhaps by different mechanisms [153, 154, 155, 156]. A study examining the synergism between PKC and PKA in the potentiation

of GSIS showed that cAMP caused bulk movement of insulin granules whereas only phorbol esters increased the number of marginated granules [156, 157]. Stimulation of this margination was lost after PKC down-regulation, suggesting that PKC-α, βII or ε might be involved. In addition, cAMP concentrations in the beta cell are increased with PKC activation probably at the level of adenylyl cyclase phosphorylation and activation [158]. This suggests the existence of cross-talk between PKC signalling and the generation of cAMP, a well-known potentiator of GSIS [159].

Phorbol esters are known to activate PKC and stimulate secretion in permeabilized beta cells where Ca^{2+} is clamped by extracellular chelation, suggesting a direct action of phosphorylation on exocytosis [160]. A number of proteins associated with the exocytotic machinery are substrates for PKC. The association of SNAP-25 or Munc-18 with syntaxin is lessened after their phosphorylation by PKC [161, 162]. Unphosphorylated Munc-18 binds to syntaxin and prevents its association with VAMP or SNAP-25, but after phosphorylation these interactions are facilitated, as is vesicle docking. The association of myristoylated alanine-rich C-kinase substrate (MARCKS) with the plasma membrane is dependent on its phosphorylation state [163]. Unphosphorylated it cross-links actin filaments at the cell periphery preventing the docking of secretory vesicles. However, phorbol-ester induced phosphorylation results in the translocation of MARCKS into the cytosol, rearrangement of cortical actin and increased vesicle docking [164]. In addition, the action of a phosphorylated cytosolic protein, P145, implicated in the Ca^{2+}-dependent catecholamine secretion was restored in PKC-deficient permeabilized PC12 cells after the addition of brain PKC [165]. Finally, recent evidence suggests that the L-type calcium channel is functionally coupled to or in close proximity with SNARE proteins in the beta cell [166, 167] and that channel function might be either augmented or inhibited by PKC phosphorylation depending on the channel's subunit composition [168, 169, 170].

Given that a phorbol ester would activate cPKC and nPKC isoforms, it is not clear yet which isoforms modulate the marginated pool of granules. It is not known whether aPKC isoforms have a similar effect on this pool, but this would be consistent with the observation that LC-CoA appears to stimulate both cPKC and aPKC isoforms [31]. The mix of DAG and LC-CoA esters at a membrane site (plasma or granule) might determine which PKC isoforms are stimulated and to what extent. In mixed micelle assays palmitoyl-CoA stimulated cPKC and aPKC activities whereas it had no effect on nPKC activity [31]. In contrast, in the presence of PS plus DAG, CoA esters of oleate and myristate partially inhibited nPKC, while stimulating aPKC activity [31]. Although the mechanism of this inhibition is not known, it was independent of DAG concentration.

Several steps in the transport of secretory granules or exocytosis machinery are candidates for modification by acylation using long-chain fatty acids. The presence of palmitoyl-CoA seems to accelerate membrane fusion processes in a reconstituted system of vesicular trafficking as it increased budding of Golgi transport vesicles from donor membranes and their fusion to acceptor cisternae [171, 172]. This action was suggested to result from protein acylation, as a non-hydrolyzable analogue of LC-CoA inhibits fusion of vesicles to Golgi surfaces. However, LC-CoA does not appear to act as a fusagen for secretory vesicles and the plasma membrane. Therefore, the observations that NEFA stimulate insulin secretion after PKC and PKA activation [52] and that in permeabilized beta cells short-term addition of LC-CoA initiates secretion [69] suggest that acylation modulates exocytosis directly.

While the core SNARE proteins syntaxin and synaptobrevin (VAMP) are thought to be associated with membranes via their hydrophobic C-termini, SNAP-25 is potentially palmitoylated at four centrally-located residues, which seems to stabilize its association with the plasma membrane [173]. However, it is not clear whether palmitoylation is required for the initial membrane targeting or only after membrane attachment of newly synthesized SNAP-25 is achieved via its association with syntaxin [174]. This latter point and the fact that palmitoylation could play a role in the proper dissociation of the core complex before membrane fusion [175] are consistent with the requirement for a functional secretory pathway in detecting in vivo acylation [176]. In addition, VAMP expressed in PC12 cells was also shown to be palmitoylated [177]. Accessory proteins, such as the Ca^{2+} sensing protein synaptotagmin and cysteine string protein are known to be acylated in situ as well [175, 178]. The acylation of the β-subunit of the L-type channel or the PKC isoform itself has been shown to enhance their function and thereby could comprise other avenues for the regulation of exocytosis by NEFA [135, 179].

Therefore, a net increase in cytosolic LC-CoA due to beta-cell stimulation by either exogenous NEFA or increased beta cell lipolysis would be a positive effector of insulin secretion, hypothesized to be mediated by changes in the distal steps in stimulus-secretion coupling.

Conclusion

The preceding sections have indicated the important roles of NEFA, LC-CoA and their esterified derivatives in affecting insulin secretion in both normal and pathological states, and the likely involvement of the various members of the PKC family of lipid-modulated protein kinases. As mentioned at the outset, the

rapid increase in obesity is the risk factor most likely to be driving the epidemic of Type 2 diabetes worldwide, and the connection between obesity and diabetes is most likely to be the high concentrations of circulating lipids and tissue triglyceride deposits, leading to increased cellular sources of NEFA. The development of Type 2 diabetes requires both beta-cell insufficiency and insulin resistance in target tissues, such that glucose disposal into muscle is retarded and hepatic glucose output is inadequately restricted. In the beta cell, chronically increased fatty acids produce inappropriate hypersecretion at low glucose concentrations, and an insufficient response to increased glucose, while in the target tissues, increased fatty acids cause insulin resistance. Interestingly, in the adipocyte, which is also an insulin-sensitive tissue, the antilipolytic effect of insulin is much less affected, so that the basal hypersecretion of the beta cell could further promote lipid storage and obesity [180]. This predicted link has been observed experimentally when inhibition of hypersecretion by diazoxide treatment was shown to enhance the weight loss observed in obese hyperinsulinaemic adults [181].

Sources. This review is based on relevant articles published in English during the period of 1990 to the present (2003) including seminal contributions prior to this. PubMed searches were done using various combinations of the following terms: fatty acid, islets of Langerhans, insulin secretion, protein kinase C, exocytosis, acylation and metabolism.

Acknowledgements. The authors would like to thank Dr. K. Tornheim for critical reading of the manuscript and to the National Institutes of Health (grants DK35914, DK63356 and DK46200) for support. Due to the journal's limit on the total number of references some pertinent references have been omitted in this review.

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