I. INTRODUCTION

Building on the pioneering studies by Coore and Randle (95) and Grodsky et al. (194) pointing to a metabolic, rather than receptor-mediated, coupling between glucose availability and insulin secretion, the “canonical” pathway of pancreatic β-cell glucose-stimulated insulin secretion (GSIS), proposed some 30 years ago (25, 94), can be summarized rather simply: “β-cells respond to raised circulating glucose with increased rates of glucose uptake and glycolysis. This, in turn, increases pyruvate delivery to the mitochondria, which increase their protonmotive force (Δψ), thus raising the cytosolic ATP/ADP ratio. This closes ATP-sensitive K⁺ channels, depolarizing the plasma membrane to the threshold at which voltage-dependent Ca²⁺ channels on the plasma membrane are activated, raising cytosolic free Ca²⁺ ([Ca²⁺]c) which in turn triggers vesicle exocytosis” (FIGURE 1). However, this straightforward textbook scheme fails to account for many of the complexities surrounding physiological insulin secretion and its dys-function in type 2 diabetes (T2D), and attempts to obtain a more precise understanding lie behind the vast subsequent literature.

There are several excellent reviews of the current consensus of the mechanisms operative in the β-cell that couple oxidative metabolism to insulin secretion (220, 255, 369, 420, 452, 460), so what is the purpose of the current contribution, particularly since it is written by an observer external to the field? The review is a response to the difficulties that this mitochondrial physiologist has encountered in trying to understand the β-cell literature in the same way as one would approach the liver, muscle, or adipose tissue. My difficulties arose in part because the β-cell field has tended to evolve in isolation from comparable studies on the cells and mitochondria of other tissues, and has consequently tended to evolve a unique terminology and approach.

An “external” scrutiny of a field has advantages but also presents problems. On the one hand, it may be more objective, since there is no personal standpoint to defend, apart from a firm belief in the Chemiosmotic Theory and the laws of thermodynamics. On the other hand, information gained almost entirely from the literature, rather than from attendance at focused meetings and conversations with leaders in the field, may mean that I place undue emphasis on published research that the field may consider flawed, or out of date, while neglecting recent key developments. Finally, I am aware that, in commenting on a number of current theories, I risk offending a number of excellent researchers; however, that is the nature and strength of science. To keep the review within bounds, I shall not review chronic adaptive changes, such as glucolipotoxicity, deficits in T2D, or receptor-mediated effects, except in so far as they impact on
the basic bioenergetics or metabolism of the cell. What remains is a vast cell biology literature, but one containing a surprising number of unsolved, or even uninvestigated, aspects.

A simplified version of the metabolic and bioenergetic pathways evaluated in this review is shown in Figure 1. To a bioenergeticist familiar with other cell types, certain aspects are notable by their virtual absence. In particular, studies with isolated mitochondria that would yield valuable information on the extent to which the β-cell mitochondrion has evolved features to fit its unique physiological role, are notable by their absence, due to the almost insuperable difficulties in purifying a homogeneous and functional β-cell mitochondrial preparation from the pancreas, free of contamination from non-β-cells and secretory granules. The complexity of upstream and downstream factors responsible for the fine control of the mitochondrial protonmotive force and the phosphorylation status of the cytosolic adenine nucleotide pool remain to be fully explored, as is a rigorous metabolic control analysis of the pathways from glucose to insulin secretion. In spite of the elegant body of research on adenine nucleotide interactions with the ATP-sensitive $K^+$ channel ($K_{ATP}$), it is sometimes difficult to relate findings to the physiological context of the intact cell. It is generally agreed that the state of many of the coupling factor hypotheses is unsatisfactory, with uncertainties over mechanisms and targets (255, 420). Finally, with notable exceptions, e.g., References 510 and 511, there appears to be limited cross-fertilization between electrophysiologists investigating ionic circuitry at the plasma membrane and biochemists focusing on the metabolism of the β-cell.

II. METHODOLOGY

A. Experimental Material

1. Primary cells

The increasing prevalence of T2D emphasizes the urgency of establishing the mechanisms underlying the dysfunctions that interfere with the normal insulin secretory response of the β-cell to changes in plasma glucose concentration. It is therefore entirely understandable that research efforts, and funding support, tend to favor experimental preparations that most closely approximate to the human disease, ideally primary β-cells and islets obtained from normal and diabetic subjects on life-support, in preference to preparations from rodents, whose islet architecture and ion channel portfolio are distinctive (64, 83). As has been often stated, “rodent diabetes is not a serious problem.” However, no two human post mortem samples have the same medical history, and while some approaches, such as the electrophysiological characterization of ion channels, that do not demand...
metabolically uncompromised tissue, provide invaluable information (64, 204), bioenergetic and metabolic studies of human samples face practical hurdles, for example, in discerning consistent differences between control and diabetic islets.

The inherent cell heterogeneity of cells in the islet (α-glucagon, δ-somatostatin, e-ghrelin, and pancreatic polypeptide cells in addition to β-cells) places significant limitations on techniques that are unable to resolve single cells and (either in real time or post hoc) establish their respective lineages. This constraint applies both in the intact islet and after dissociation into single cells, and is probably most serious in metabolic studies that require entire populations of cells.

**2. Insulinoma cell lines**

The literature cited in this review is mostly drawn from studies on intact islets, dissociated primary β-cells and insulinoma cell lines, these last being even further removed from human pathophysiology. It is therefore frequently asserted that studies utilizing cell lines are of dubious relevance to understanding the basic mechanisms of insulin secretion. However, such a conclusion presumes that our knowledge of the fundamental mechanisms inherent in glucose-sensing, insulin-secreting, cell preparations is essentially complete, and that the field possesses a sufficient vocabulary to detect aberrations in conditions of human diabetic pathology. A central theme of this review, seen from the perspective of cellular bioenergetics, is that this stage has yet to be attained, and that information concerning the normal metabolic and bioenergetic functions of insulin-secreting cells, and how these functions differ from cells in other tissues, is incomplete. To draw an analogy, *Drosophila* and *Caenorhabditis elegans* are considered valid models for understanding mechanisms applicable to human aging over a vastly greater phylogenetic chasm than that between human and rodent islets and appropriate insulin-secreting cell lines.

A multiplicity of insulin-secreting cell lines have been derived over the past 35 years, in attempts to establish models that retain the key features of primary rodent, and ultimately human, β-cells, including a physiologically relevant content of secretory granules, metabolic and secretory responsiveness to glucose and expression of plasma membrane ion channels (reviewed in Ref. 485). It is fair to say that, to date, none of these attempts has been fully successful. From the bioenergetic standpoint of the present review, essential characteristics include the retention of low-affinity glucose transporters and hexokinase 4 (glucokinase), insulin biosynthetic pathways, ion channels and exocytotic machinery, and low or absent expression of lactate dehydrogenase (477). This last performs multiple functions, firstly, as in the primary β-cell, it ensures the absence of a Pasteur effect, whereby accelerated glycolysis can compensate for decreased oxidative phosphorylation when the latter is compromised. The lack of lactate dehydrogenase could actually increase the bioenergetic “efficiency” of a cell in which glucose delivery may be limiting, by preventing anaerobic glycolysis (2 ATP per glucose) and ensuring stoichiometric oxidative phosphorylation (20-30 ATP per glucose, depending on proton leaks). The second, related, consequence is that the Warburg effect (transition to a glycolytic phenotype) is prevented in cell lines that display low expression of lactate dehydrogenase, with the result that they retain a strong oxidative metabolism, despite being proliferating cells. In primary β-cells, the low expression of lactate dehydrogenase, together with the absence of a plasma membrane monocarboxylate transporter, ensures that circulating lactate and pyruvate cannot trigger inappropriate insulin secretion during exercise (235, 425).

The most widely used insulinoma cell lines have been derived from a transplantable islet tumor induced in an NEDH (New England Diaconess Hospital) rat by high-dose X-irradiation (169). The original cell line, RINm5F, is still occasionally used, but in 1992, Asfari et al. (24) used RINm5F cells to establish a new cell line (INS-1). Concerns over their variability led to the creation of a number of INS-1 derived clones, including INS-1E (347). In 2000, Hoehmeier et al. (226) stably transfected INS-1 cells with a plasmid containing the human proinsulin gene, and derived the strongly responsive INS-1 832/13, together with poorly responsive clones such as 832/2 (24). A human-derived β-cell line, EndoC-βH1, has been developed (433), using targeted lentiviral oncogenesis in human fetal tissue, and characterized (17, 197, 433). These cells possess the advantage that their proteins are encoded by human genes, overcoming the criticism that the ion channels involved in generating plasma membrane potential responses differ between rodent and human cells. Also commonly employed are MIN6 cells, established from insulinomas produced by the targeted expression of the simian virus 40T antigen gene in transgenic mice (355). Less common are the BRIN BD11 cell line established by electroporation of RINm5F cells with primary rat islet cells (341) and βHC9 cells, derived from islet cells of mice expressing the SV40 T antigen under the control of the insulin promoter (451). Finally, progress is being made towards generating functional β-cells from human embryonic stem cells (396).

A limitation in the investigation of any proliferating cell line is that their metabolism is adapted for continuous cell division, and thus does not mimic mature primary β-cells that proliferate either very slowly or not at all. They therefore funnel more anaplerotic metabolism and ATP demand into the synthesis of the proteins, phospholipids, and metabolites required for cell growth. The human-derived EndoC-βH2 cells (470) possess excisable immortalizing transgenes allowing for inhibition of cell proliferation. While these hold promise, to date they have not revealed any novel features.
In attempting to review the characteristic bioenergetic behavior of β-cells, it is necessary to distinguish features that appear to be common across primary cells and cell lines, and are thus likely to provide generic information on the inherent features of a glucose-responsive, insulin-secreting cell, from those that are only apparent in a single cell line, and are thus likely to represent epiphenomena unessential for the canonical pathway of GSIS. An exception is the ability of many cell lines, in contrast to primary β-cells, to utilize exogenous pyruvate as substrate, which provides a valuable tool to investigate metabolism and bioenergetics while bypassing glycolysis.

3. Insulin secretion protocols

The ultimate goal of most β-cell research is to understand the mechanisms underlying GSIS, and to detect deficits of relevance to T2D. In humans, a fasting plasma glucose concentration of 7 mM or higher, together with an HbA1c (glycosylated hemoglobin A1c) in excess of 6.5%, indicates T2D. This may be backed up by an oral glucose tolerance test (OGTT), in which 75 g glucose is given to fasting subjects, and plasma glucose is determined 2 h later, with a final value of 11 mM or higher supporting the diagnosis of T2D. A full time course over 3 h of plasma glucose levels during an OGTT of a healthy subject (66) shows a smooth rise and subsequent recovery. Typically, plasma glucose slowly increases from 5.5 to 9.5 mM over 60 min and returns to basal over the subsequent 3 h (FIGURE 2A). In healthy subjects, the rate of insulin secretion, facilitated by the secretion of gut-derived incretins (130), closely mirrors the glucose concentration.

In contrast, the majority of in vitro studies employing isolated islets, dissociated primary cells or insulinoma cell lines, have employed a simplified protocol involving a step increase in extracellular glucose, typically from 2.8–3 to 11–16 mM or even higher. Under these rather extreme conditions, perfused, intact, islets show a biphasic release of insulin, with an initial rapid and transient release followed by a slowly accelerating secretion, particularly at supramaximal glucose (FIGURE 2B). The two phases are commonly referred to as first (or triggering) and second (or amplification) phases (220). However, as emphasized by Prentki et al. (420), “this biphasic pattern does not exist in vivo under physiological conditions in which blood glucose rises relatively slowly after a meal.” It may be significant that, at least in some studies (108), biphasic release from human and mouse islets in response to high glucose is not seen when the islets are preincubated in a more physiologically relevant 5.6 mM glucose. Thus, to some extent, many in vitro studies have exploited a nonphysiological condition, albeit one that has provided a wealth of information by exposing multiple components that would not normally be rate-restricting, but which respond with differing kinetics to the sudden elevation in glucose.

Interpretation of the time course of insulin secretion would be more straightforward if the upstream metabolic and bioenergetic parameters responded instantaneously to the sudden glucose transition, in much the same way that the cardiac muscle cell responds to an increased work load. However, after starvation in low glucose, the β-cell requires up to 60 min to achieve a new metabolic and bioenergetic
steady state following a step elevation in glucose, with respiration (184), cytosolic ATP/ADP ratios (509), and metabolite levels (491), each showing different recovery kinetics. This greatly complicates interpretation of the resulting insulin release profile. Bypassing these upstream events and monitoring exocytosis directly from the increase in cell capacitance in response to imposed square-wave depolarization indicates that vesicle trafficking and docking can account in part for the biphasic response. A rapid, ATP-independent, release of a small pool of vesicles is followed by a slower ATP-dependent secretion (Figure 2C), consistent with a distinction between “docked” and “reserve” vesicle pools (453, 454). It should be noted, however, that multiple additional factors, in particular mediated by cAMP, are implicated in the control of insulin granule dynamics, as will be discussed later (482).

The rate of second phase insulin secretion increases with time during continuous exposure of intact islets to elevated glucose. A vast literature has been devoted to attempts to understand the mechanisms underlying this biphasic response, with the elaboration of theories requiring amplification by metabolic coupling factors (e.g., Refs. 218, 255, 322, 420, 548). The division of in vitro insulin secretion into two mechanistically distinct phases originated with the observation that glucose appeared to have additional effects on insulin secretion beyond $K_{\text{ATP}}$ closure (reviewed in Ref. 219). In particular, the group of Henquin (172) described an alternative mode of insulin release from mouse islets, in which $K_{\text{ATP}}$ channels were locked open by diazoxide, and external $K^+$ was increased to 30 mM. The majority of insulin release was delayed in this protocol, and this “$K_{\text{ATP}}$-independent” insulin secretion was originally proposed to model the second, amplifying phase, although the distinction between first and second phase release is now considered to be more blurred (219). Some insulin release occurred in 3 mM glucose, but was increased two- to threefold by 20 mM glucose (218). There is a tendency in the field to overlook the vast number of ATP-dependent processes in the $\beta$-cell additional to $K_{\text{ATP}}$ closure, and the most economical explanation for the retained glucose dependency is that an elevated cytosolic ATP/ADP ratio is required for one or more additional process associated with exocytosis, such as the priming and transport of reserve vesicle to the sites of exocytosis (453). Thus Detimary et al. (122) titrated mouse islets stimulated with high glucose or glucose/KCl/diazoxide with a range of mitochondrial inhibitors and concluded that a step distal to $[\text{Ca}^{2+}]_c$ elevation required an elevated ATP/ADP ratio to support secretion. The role of the cell’s parallel energy-sensing pathway, AMP-kinase, will be discussed later.

Electrophysiology has revealed a wealth of $Na^+$, $Ca^{2+}$, and $K^+$ ion channels on the $\beta$-cell plasma membrane with distinctive activation and inactivation kinetics responsible for the complex oscillatory behavior of the plasma membrane potential once the dominant $K_{\text{ATP}}$ channel is inhibited (64, 133, 452). There is persuasive evidence that the frequency and magnitude of these oscillations are central to understanding the control of insulin secretion, since oscillations, as well as first phase release, are defective in T2D (393). One concern is that the high $K^+$/diazoxide protocol clamps $\Delta\psi_m$ at an invariant depolarized potential, with the result that the subtleties of oscillatory control are over-ridden. A final, related, concern is that many metabolism-centered investigations attempting to correlate metabolic status to insulin secretion in dissociated cell populations tend to overlook the role of ionic oscillations, and the fact that these are stochastic and heterogeneous (175, 414, 481).

In conclusion, it is helpful to bear in mind that the conventional step transition from low to high glucose, and the use of elevated KCl in the so-called $K_{\text{ATP}}$-independent mode of insulin secretion, are in vitro constructs that do not necessarily reflect the in vivo responses.

### III. BIOENERGETIC BASIS OF GLUCOSE-STIMULATED INSULIN SECRETION

The classic textbook explanation for the unique ability of the $\beta$-cell to respond so sensitively to physiological variations in plasma glucose concentration is that the cell’s possession of a low-affinity ($K_m$ 15–20 mM) glucose transporter [GLUT-2 in the mouse, mainly GLUT-1 and GLUT-3 in human (114) although GLUT-2 is also detected (108)], together with the low-affinity hexokinase IV (glucokinase), ensure that glycolytic flux, and ultimately the cytosolic ATP/ADP ratio, is responsive to physiological variations in glucose availability. This is clearly an insufficient explanation, since hepatocytes also possess glucokinase and GLUT-2 (albeit under insulin control) without demonstrating a similar bioenergetic sensitivity to glucose.

In the $\beta$-cell, the downstream dissipative pathways that balance the upstream delivery of glucose-derived pyruvate to the mitochondrial are of critical importance, but are commonly neglected. These include the abnormally high endogenous mitochondrial proton leak (2, 542) and the largely unexplored quantitation of ATP utilizing pathways (but see Refs. 178, 502). In the absence of these dissipative pathways, even the slowest trickle through the glycolytic pathway would eventually result in a maximal ATP/ADP ratio approaching thermodynamic equilibrium. It is therefore important to understand how the $\beta$-cell, which has evolved to vary the Gibbs free energy of the cytosolic ATP hydrolysis reaction ($\Delta G_p$) as a function of upstream substrate availability, differs from, for example, heart muscle, which possesses feedback mechanisms to ensure that $\Delta G_p$ remains as constant as possible in response to huge variations in ATP demand.

To understand this distinction, it is necessary to review some basic thermodynamic and kinetic principles gov-
erning cellular bioenergetics. Any energy transfer system has intensive (“force”) and extensive (“flux”) parameters. Examples of the former include the pressure of the water flowing into a hydroelectric turbine, electrical voltage, protonmotive force (Δp) or ΔGp. “Extensive” parameters include the rate of water flow through a turbine, electrical current, respiratory rate, and the rate of ATP turnover. A simplistic hydraulic analogy can be used to illustrate that skeletal muscle and the β-cell are, in terms of metabolic control, at opposite ends of a continuum. We can crudely model the relationship between energy flux (an extensive parameter) and potential (intensive) in the skeletal muscle cell by imagining a water container with leaks representing ATP utilization and proton leak and an input that senses the water level, for example, by a float valve (Figure 3). The height of the water in the container represents Δp.

The mitochondria in muscle and muscle-derived cells are notable for possessing an endogenous proton leak that is not only low, but also “nonohmic”, i.e., highly potential-dependent (2), allowing the proton current to be efficiently utilized for ATP synthesis. This is modeled by placing the leak high up on the side of the container, so that it is active in resting “muscle” (Figure 3Ai), but stops when the water level (“Δp”) falls during muscle contraction. ATP utilization during muscle contraction is modeled by opening the drain tap (Figure 3Aii), lowering the water level (Δp). This in turn feeds back to open the input valve, largely, but not completely, restoring the water level. Thus, in the muscle cell, any drop in Δp, and hence ΔGp, is immediately sensed by the glycolytic pathway, which accelerates in an attempt to restore the phosphorylation potential. In reality, the drop in ΔGp is further minimized by Ca2+–mediated upstream activation of metabolism (a further opening of the upstream

![Figure 3](http://physrev.physiology.org/)
stand the control of dissipative, pathways and the glycolytic input to underestimate that it is important to consider both the downstream, reduce a straight line). These crude models attempt to illustrate that high glucose roughly doubles respiration (FIGURE 3D, i.e., roughly proportional to the height of the water column, which is why it is placed at the bottom of the container. Basic “housekeeping” ATP turnover also responds to “Δp,” which stabilizes at a suboptimal level at which the restricted input corresponding to low glucose balances the sum of the two outputs (FIGURE 3B). Cell respirometry indicates that high glucose roughly doubles respiration (FIGURE 3D) (17, 147, 184, 331). To model this, we double the rate of water inflow (FIGURE 3Bi), and as a result, the water level rises until the increased hydrostatic head has increased the two outflows sufficiently to balance in the input. In the real β-cell, the resulting Δp produces a ΔGp sufficient to inhibit the K+ATP channel and trigger insulin secretion.

The respiratory data in FIGURE 3C were taken from a representative cell respirometer experiment (331). Respiration of INS-1 832/13 cells measured after 60-min incubation in the presence of 2.8 or 16.7 mM glucose reflects the total proton current through the proton leak and ATP synthase. Addition of the ATP synthase inhibitor oligomycin restricts proton reentry to the leak. The drop in proton current (respiration) with oligomycin is sometimes taken to represent the current through the ATP synthase prior to addition of the inhibitor, although, strictly, any changes in Δp must be allowed for. FIGURE 3D shows a current-voltage relationship for the endogenous proton leak of mitochondria isolated from INS-1E cells (2). The mitochondria respiring on succinate maintained a characteristic Δp and respiratory rate in the absence of ATP synthesis, a condition referred to as “state 4.” The only significant pathway for proton reentry under these conditions is the proton leak. To determine how the leak current varies with voltage (Δp), succinate dehydrogenase was progressively inhibited with malonate, with current and voltage determined at each step. The leak is nearly ohmic over this range (an ohmic conductance would produce a straight line). These crude models attempt to illustrate that it is important to consider both the downstream, dissipative, pathways and the glycolytic input to understand the control of ΔGp in the β-cell.

A. Metabolic Control Analysis

As naively modeled in the preceding section, β-cells respond to an increased glucose level by accelerating the rate of metabolism (an extensive, “flux”, parameter), while the increased flux controls the level of a key intermediate, Δp (an intensive, “force”, parameter). This control is central to an understanding of GSIS, and its investigation falls within the remit of metabolic control analysis (MCA). MCA has been developed for the quantitative determination of the extent to which individual processes within a reaction pathway control the flux and concentrations of intermediates. There are several variations of MCA, extensively covered in reviews and texts (8, 100, 101). The importance of MCA in drug development, for example, is that it helps to identify targets that exert significant control over the metabolic pathway investigated (360). The extensive literature employing MCA to investigate the control of glycolysis and oxidative phosphorylation in skeletal muscle (reviewed in Ref. 274) and in proliferating cancer cell lines (358, 359) attests to the importance of understanding multiple control points in the metabolism of these cells. It is necessary to emphasize that metabolic conclusions based on gene expression or enzyme content are generally invalid, unless accompanied by evidence that the affected step exerts a significant control over flux or concentration.

Metabolic control analysis of a complex pathway, such as that linking glucose to the cytosolic adenine nucleotide pool, can be simplified by grouping individual processes into modules, and considering each module as a black box with an input and output linked to other black boxes by an intermediate, such as ATP. This has been termed “top-down” or modular kinetic analysis (58, 270). Modular kinetic analysis has been performed on isolated mitochondria (7, 58), including those from INS-1E cells (2). However, in view of the universal consensus that glycolysis, mitochondrial metabolism, and the cytosolic adenine nucleotide pool are central to the understanding of normal and pathological (diabetic) function of the β-cell, it is notable that I can locate no complete metabolic control analysis of these pathways in the intact cell.

The first principle of MCA is that one never encounters a strictly “rate-limiting” process. Instead, control is distributed throughout the pathway. “Control” has a precise meaning in MCA; the flux control coefficient of a step in a metabolic pathway is defined as the fractional change in flux through the entire pathway produced by a (small) fractional change in the activity of the step, whether caused by an inhibitor or activator. Flux control coefficients are therefore employed to investigate the control of extensive parameters (rates of glycolysis, respiration, ATP turnover, etc.). For any normal pathway, the sum of all flux control coefficients equals 1, so it is possible to determine how control is apportioned at each step in the pathway. For example, in the glycolytic pathway, a high flux control coefficient at glucokinase cannot coexist with a high coefficient at phosphofructokinase. Questions that might be addressed include establishing which steps in the entire sequence from glucose to the citric acid cycle exert significant flux control under low and high glucose conditions.
The control of intensive variables (substrate concentration, $\Delta p$, $\Delta G$, etc.) can be investigated by determining concentration control coefficients. The concentration control coefficient of a step in a pathway is defined as the fractional change in the steady-state concentration of an intermediate in response to a small fractional change in activity of the process for which the metabolite is a substrate or product. While the above coefficients apply to components of complete pathways, elasticity refers to the way in which the rate of an individual step in a pathway responds to a small change in the concentration of its substrates, products, activators, or inhibitors. If an enzyme responds to a 2% increase in substrate concentration with a 0.4% increase in rate, the elasticity coefficient ($\varepsilon$) will be 0.4/2 or 0.2. For simple reactions, elasticity coefficients are related to substrate concentration and $K_m$, approaching 1 at very low concentration, 0.5 at the $K_m$ and 0 at saturating substrate.

As an example, ATP utilization by the contraction of actomyosin fibers in a muscle cell is relatively insensitive to changes in ATP/ADP ratio (the process has a low elasticity) but a high flux control (it largely defines the rate of ATP turnover). In the $\beta$-cell, the key downstream event ($K_{ATP}$ closure) has a high elasticity towards (is highly responsive to) the ATP/ADP ratio, but exerts little flux control (has little effect on ATP turnover), whereas key upstream steps, such as glucokinase and PFK, show high elasticity to the ATP/ADP ratio, low flux control, and high concentration control over ATP/ADP. One consequence of this last is that intermediate levels in the $\beta$-cell are allowed to vary, contributing to the massive changes in glycolytic and citric acid cycle intermediate levels during a low-to-high glucose transition (see Figure 7).

Without some form of MCA, the simple canonical model of GSIS fails to explain how insulin secretion is so precisely controlled, since alterations in substrate delivery, mitochondrial metabolism, and subtle alterations in proton leak or ATP turnover can disturb the cytosolic ATP/ADP ratio, and hence the delicate balance between plasma glucose and insulin secretion. Finally, to reiterate, it’s just as important to investigate the downstream dissipative pathways as the intensely investigated upstream, glycolytic pathways to understand the control of $\beta$-cell metabolism.

### B. Isolated Mitochondria

In a “normal” tissue, the classical approach to the investigation of cellular bioenergetics would include a strong emphasis on the properties of the isolated mitochondria or an equivalent permeabilized cell preparation. The utility of isolated mitochondrial studies lies in the ability to establish the extent to which unique metabolic features of a cell can be ascribed to the mitochondria themselves, rather than their cellular environment. Such studies provide the backbone to our understanding of the metabolism of many tissues, providing information on respiratory capacity, ATP synthesis, proton leaks, metabolite access via inner mitochondrial membrane transporters, control over the utilization of pyruvate, fatty acids, and amino acids etc. The isolated mitochondrial preparations that have been studied in depth, such as those from liver, heart, skeletal muscle, brown adipose tissue, insect flight muscle, etc., each display characteristic transport and bioenergetic properties adapted to their physiological roles.

This approach has been immensely powerful in thousands of publications, facilitating subsequent integration of isolated mitochondrial findings into whole cell physiology. Unfortunately, such an approach is of limited practicality for primary $\beta$-cell mitochondria, located within islets, themselves comprising a tiny fraction of the entire pancreas. Thus the 1 million $\beta$-cells in a mouse account for $<$1% of the mass of the pancreas (52), while many of the cells within the islet are not $\beta$-cells. Indeed, the only functional studies on islet mitochondria of which I am aware isolated minute quantities of mitochondria from $ob/ob$ mouse islets and used the sensitive luciferase assay to detect oxidative phosphorylation, since the low yield of material precluded conventional techniques, such as respiratory (233, 289, 290). The greater yield of cells from insulinomas, either obtained directly from the experimental animal (91, 418), or from established cell lines (91), have allowed a very limited number of classical isolated mitochondrial studies to be performed, as well as assays of enzyme levels in mitochondria isolated from insulinoma cells (71, 312). It is notable that INS-1 cells (459) and primary $\beta$-cells (477) possess unusually high mitochondrial $\alpha$-glycerophosphate dehydrogenase activity.

The few isolated mitochondrial studies that exist provide hints of unusual properties. Thus, while pyruvate is clearly the major physiologically relevant substrate in the intact cell, for unexplained reasons the isolated mitochondria appear to have difficulty oxidizing NAD-linked substrates, the failure being either stated explicitly (2), or inferred by the authors only reporting data obtained with substrates bypassing complex I, such as $\alpha$-glycerophosphate and succinate (137, 269, 276, 324, 426). The basis of this failure, which has also been reported for permeabilized $\beta$-cells (see below) merits further investigation, since it may suggest that a critical component is lost. Exceptions exist; thus a study by Civelek et al. (91) showed that mitochondria isolated from an in situ rat insulinoma were able to oxidize 2-oxoglutarate, glutamate, or pyruvate in the presence of malate, while mitochondria isolated from the insulin-secreting $\beta$-HC9 cell line oxidized pyruvate plus malate (128).

### C. Permeabilized Cell Preparations

Selective permeabilization of the plasma membrane removes the barrier preventing impermeant substrates from
accessing the mitochondrion, while avoiding the technical problems associated with the separation of small amounts of mitochondria by classical differential centrifugation (289, 291). It is, of course, essential that this is performed in a mitochondria-friendly medium containing submicromolar free Ca\(^{2+}\). Permeabilization of insulinoma cell lines has been performed with saponin (89, 91, 96, 98, 139, 269), staphylococcal α-toxin (246, 323, 324, 326), streptolysin-O (117), or perfringolysin-O (260) or by electropermeabilization (459). The advantages and limitations inherent in these permeabilization strategies have been recently reviewed, with a focus on mitochondrial function in skeletal muscle (408).

As with the isolated β-cell mitochondria, substrates feeding directly into the ubiquinone pool, such as α-glycerophosphate and succinate, are much more effective substrates for permeabilized β-cells than those involving complex I, and are used almost exclusively (e.g., Refs. 233, 269, 323, 326, 427, 459). Maechler et al. (323) reported that methyl-pyruvate (rather than pyruvate) supported matrix Ca\(^{2+}\) accumulation and insulin secretion from α-toxin-permeabilized INS-1 cells (323). However, methyl-pyruvate has been proposed to influence insulin secretion by a mechanism other than as a mitochondrial substrate (291). Kim et al. (260) permeabilized the plasma membranes of INS-1E and hamster embryonic kidney 293 (HEK293) cells utilizing recombinant perfringolysin-O (nPFO). Consistent with the isolated β-cell derived mitochondria, the permeabilized INS-1E cells showed robust state 3 respiration (i.e., in the presence of ADP) with either succinate or glycerol-3-phosphate, but failed to respond to malate in combination with glutamate, pyruvate, isocitrate, or 2-oxoglutarate, classic substrates oxidized by the citric acid cycle in cellular bioenergetics. It is instructive to consider the analogy between the proton circuit and an equivalent simple electrical circuit (382). Both possess intensive (voltage and protonmotive force Δp, respectively) and extensive (electrical current, proton current) terms. The protonmotive force is described in electrical units (mV), while Ohm’s Law (current = voltage/resistance) can be applied to both circuits, and current-voltage relationships determined, particularly for the endogenous proton leak (FIGURE 3C). As in an electrical circuit, an increase in the conductance of a proton reentry pathway leads to an increase in current and a drop in voltage.

The protonmotive force across the inner mitochondrial membrane has electrical (membrane potential, Δψ) and chemical (transmembrane pH gradient, ΔpH) components

\[ \Delta p (\text{mV}) = \Delta \psi - 61 \Delta \text{pH} \]

The negative sign results from the original sign convention (an acidic matrix corresponding to a positive ΔpH). To distinguish plasma and mitochondrial membrane potentials, we shall henceforth use the respective symbols Δψ\(_m\) and Δψ\(_p\).

1. Mitochondrial membrane potential (Δψ\(_m\))

Techniques for quantifying the components of Δp in isolated mitochondrial preparations have been long established (354, 373). However, the virtual absence of information on isolated β-cell mitochondria has restricted investigations to the more complex and less defined context of the intact cell. Exogenous fluorescent membrane-permeant cations, such as tetramethylrhodamine methyl ester (TMRM), accumulate into the cytoplasm of intact cells as a function of Δψ\(_m\), and from the cytoplasm to the matrix as a function of Δψ\(_m\) (378, 533). While it is in theory possible to estimate Δψ\(_m\) by confocal microscopy from the ratio of fluorescence intensity between individual mitochondria and the surrounding cytoplasm (see Refs. 288, 511), errors in quantification are introduced by the inability to resolve a mitochondrial matrix free of surrounding cytoplasm, movement of mitochondria out of the focal plane, and technical difficulties in quantifying a >100-fold ratio of fluorescence intensity between mitochondria and cytoplasm (78). The technique has, however, proven fruitful in the analysis of Δψ\(_m\) heterogeneity in β-cells and the relation to the fission/fusion cycle and quality control (521). The problem of mitochondrial motility can be solved by inclusion of a second, potential-independent, mitochondrial probe and ratioing of the emission (521).

A more common technique to monitor changes in Δψ\(_m\) is to image at single-cell resolution, determining total cell fluo-
resence of a cationic probe such as TMRM (173, 183), or the more slowly equilibrating rhodamine 123 (R123) (135, 286, 304). This technique is complicated by the plasma membrane potential, $\Delta \psi_p$, which controls the gradient of the probe between the incubation medium and the cytosol. This is particularly important in the case of the $\beta$-cell, where $\Delta \psi_p$ responds to changes in cytosolic ATP/ADP ratio generated by changes in $\Delta \psi_m$. Adding to the complexity, the relationship between the single cell fluorescence and $\Delta \psi_m$ differs when the cell is equilibrated with low nanomolar concentrations of TMRM, allowing the probe to remain unaggregated and fluorescent in the matrix (so-called non-quench mode) as opposed to equilibration with $>$50 nM probe, when aggregation causes quenching of the fluorescence in the matrix (“quench mode”) (reviewed in Ref. 61). Briefly, quench mode is a sensitive means to detect relatively rapid changes in $\Delta \psi_m$ complete within 1–2 min during the experiment. Changes in $\Delta \psi_m$ and $\Delta \psi_p$ can generally be distinguished by both direction and kinetics (380): mitochondrial hyperpolarization resulting in a rapid partial quenching as further probe accumulates into the quenched environment of the matrix, while redistribution across the plasma membrane in response to changes in $\Delta \psi_p$ is far slower, particularly in the case of R123, and can generally be neglected in short-term experiments.

In contrast, nonquench mode (e.g., Refs. 173, 174, 211) is more quantifiable, but the probes respond slowly and are affected equally by changes in $\Delta \psi_m$ and $\Delta \psi_p$. Changes in $\Delta \psi_p$, which are particularly relevant in the $\beta$-cell context, can be allowed for by including a fluorescent anion together with TMRM (173, 174, 378). These approaches have been recently reviewed (61, 380). Formerly, these have been either qualitative (“mitochondria hyperpolarize or depolarize”) or semi-quantitative (with estimates of the extent of the change in $\Delta \psi_p$, in transitioning from low to high glucose, Ref. 183); however, a novel technique employing simultaneous cationic and anionic probes has allowed quantitative determination (173, 174).

2. Mitochondrial $\Delta \psi$

While $\Delta \psi_m$ is the dominant component of $\Delta \psi$, there is the unfortunate tendency, with some notable exceptions (12, 536), to ignore the contribution of $\Delta \psi$ and equate $\Delta \psi_m$ with the full $\Delta \psi$. Broadly speaking, the uptake of charged species, such as Ca$^{2+}$, allows $\Delta \psi$ to increase at the expense of $\Delta \psi_m$, while that of phosphate tends to discharge $\Delta \psi$ (376). We shall see below how these factors come into play in $\beta$-cells exposed to high glucose. Determination of mitochondrial $\Delta \psi$ has been achieved in intact and dissociated rat islets by infecting cells with an adenovirus carrying the mitochondrial targeted yellow fluorescence protein (YFP) variant mtAlpHHi (1) that responds linearly to pH over the range from 7.0 to 8.5 (536). The parallel determination of cytosolic pH, using the ratiometric fluorescent probe BCECF, has allowed $\Delta \psi$ to be quantified under differing metabolic conditions (12, 536).

E. How Does the $\beta$-Cell Protonmotive Force Respond to Glucose?

A qualitative mitochondrial hyperpolarization in response to elevated glucose was first reported by Duchen et al. in single dissociated mouse $\beta$-cells, utilizing R123 in quench mode (135). In addition to glucose, a variety of plasma membrane-permeant mitochondrial substrates are able to hyperpolarize the $\beta$-cell mitochondria, including $\alpha$-ketoisocaproic acid (135, 400), methyl-succinate (286, 323) and, in INS-1 derived cells possessing the plasma membrane monocarboxylate carrier, pyruvate (183). A semi-quantitative estimate of the mitochondrial hyperpolarization in INS-1 832/13 cells gave an estimate of 14 mV on increasing glucose from 3 to 16 mM, while 9 mM pyruvate produced a more rapid 16 mV hyperpolarization (FIGURE 4C) (183).

At high resolution, individual mitochondria within the $\beta$-cell can be imaged in the confocal microscope (541). Ratiometric imaging of dispersed primary $\beta$-cells with TMRM (in nonquench mode), together with the largely $\Delta \psi_m$-independent MitoTracker Green, allowed compensation for movement out of the focal plane. A considerable heterogeneity in relative $\Delta \psi_m$ was observed in individual cells, while increasing glucose from 3 to 8 mM resulted in a mean hyperpolarization of 6 mV (541). With appropriate controls and calibration, it has been possible to obtain quantitative measurements of $\Delta \psi_m$ using TMRM in combination with an oxonol-type $\Delta \psi_p$ indicator, “PMPI” (174). Application of this technique to a limited set of nondiabetic human primary $\beta$-cells indicated a $\Delta \psi_m$ of 124–138 mV in 3 mM glucose, increasing to 152–174 mV when glucose was raised to 16 mM (173).

It must be reemphasized that even a quantitative estimate of $\Delta \psi_m$ hyperpolarization cannot be equated with a change in $\Delta \psi$ unless the $\Delta \psi$ component is also determined (12, 536). Wiederkehr et al. (536) determined matrix and cytosolic pH in low and high glucose for rat islets and INS-1E cells. In the former, $\Delta \psi$ increased from $-0.13$ units in 2.5 mM glucose to $-0.51$ units in 16.7 mM glucose, probably as a consequence of Ca$^{2+}$ uptake into the matrix (FIGURE 4D). This implies that changes in $\Delta \psi$ may contribute more than 20 mV to the total $\Delta \psi$ hyperpolarization, comparable to the $\Delta \psi_m$ hyperpolarization. Combining the data for $\beta$-cell $\Delta \psi_m$ and $\Delta \psi$ suggests that $\Delta \psi$ may be capable of increasing from $\sim140$ to $\sim180$ mV during the transition from low to high glucose, although there remains considerable uncertainty in these values, and more precise determinations are required.
F. Proton Current

Proton current, an extensive function, is surprisingly simple to quantify, both in isolated mitochondria and intact cells. The electron transport chain has a precise stoichiometry between electron flow and proton translocation, with the accepted stoichiometry being 10 $H^+$/H$_2$O translocated per 2 $e^-$ passing from NADH to O$_2$, and 6 $H^+$/H$_2$O for a substrate such as succinate or $\alpha$-glycerophosphate entering at the level of the UQ pool (382). After correction for any nonmitochondrial oxygen uptake, the total proton current in the preparation is thus directly proportional to the respiratory rate. The technical requirement is for a sensitive means to quantify the uptake of oxygen by isolated islets or attached primary or insulinoma $\beta$-cells. Early studies employing a conventional Clark-type oxygen electrode required up to 100 islets and was able to detect a glucose-dependent increase in respiration (90, 399, 401). Jung et al. (250) used an oxygen micro-sensor to detect oscillating oxygen demand in single islets, while Sweet et al. (503) employed a perfusion technique.

G. Cell Respiratory Control

The advent of the Seahorse Respirometer has allowed the respiration of small numbers of islets (542) or attached $\beta$-cells (331) to be monitored. The standard cell respiratory control (CRC) protocol, involving the sequential additions of oligomycin and a protonophore such as FCCP (61), can yield information on basal respiration, the proportion of the proton current utilized for ATP synthesis and the endogenous proton leak, and maximal respiratory capacity (FIGURE 4A). Measurements can be performed in low glucose, high glucose, and during the transition between the two states. To a first approximation, the acute decrease in respiration on adding oligomycin is a measure of the proton current through the ATP synthase prior to the addition of the inhibitor and is hence a measure of the rate of mitochondrial ATP synthesis, while the residual respiration in the presence of oligomycin is taken as a measure of the endogenous proton leak. It must, however, be borne in mind that, depending on experimental conditions, additional proton utilizing pathways may contribute to the observed oligomycin-insensitive respiration. Thus the exchange of cytosolic ADP and $P_i$ for ATP via the adenine nucleotide translocator and phosphate transporter is driven by the proton circuit, while additional minor proton reentry pathways include the Na$^+$/H$^+$ antiporter driving calcium cycling via the Na$^+$/Ca$^{2+}$ antiporter and the Ca$^{2+}$ uniporter (377), and the malate aspartate shuttle transferring electrons from cytosolic to matrix NADH pools.

1. Coupling efficiency

Coupling efficiency is strictly defined as the fraction of the total proton current, at constant $\Delta p$, that is coupled to ATP synthesis (rather than to leak pathways). Since the addition of oligomycin causes a mitochondrial hyperpolarization, the residual oligomycin-resistant respiration will produce an overestimation of the proton leak prior to addition of the inhibitor (5). The correction can be almost 50% if the cell’s
mitochondria were close to state 3 prior to oligomycin (figure 3c), but is generally 10–30% in cells during “basal” ATP turnover. Precise coupling efficiencies are determined at constant Δp, requiring at least qualitative estimates of this parameter. There is general agreement that β-cell mitochondria possess a high endogenous proton leak (5, 17, 163, 542), consistent with the “leaky bucket” analogy discussed above.

2. Respiratory capacity

The final stage in a CRC protocol is the addition of a carefully calibrated concentration of protonophore, typically FCCP, just sufficient to eliminate respiratory control. Excessive depolarization tends to reverse the ΔpH across the inner membrane, opposing the uptake of pyruvate from the cytosol. The addition provides an indication of the maximal respiration achievable with the given substrates. However, the accuracy of the CRC experiment relies on the ability of glycolysis to accelerate sufficiently in the presence of oligomycin to maintain cellular ATP levels, since collapsing ATP would inhibit glycolysis with its two ATP-dependent metabolic steps. In the generic cell, the acceleration of glycolysis in response to a lowered ΔGp is generally sufficient to maintain ATP levels for the duration of the experiment. In the case of the β-cell, with its restriction on anaerobic glycolysis due to the low levels of lactic dehydrogenase and/or the monocarboxylate transporter, such compensation cannot occur, with the result that ATP depletion may limit the respiration achieved in the presence of the protonophore (184, 306, 531). To overcome this limitation, a separate experiment, involving the addition of oligomycin together with FCCP, allows maximal respiration to be monitored prior to ATP depletion.

G. The Endogenous Proton Leak

The endogenous proton leak is distinct from that hypothesized to be associated with UCP2. Indeed, even after several decades of investigation, the molecular identity of the endogenous leak remains unclear. Correlation studies have suggested an association with the adenine nucleotide transporter ANT (62, 238), although β-cells are reported to show low expression of ANT (2) and yet possess a high leak. The current-voltage relationship of the leak in isolated mitochondria (i.e., the relationship between state 4 respiration and protonmotive force) can be determined by progressively restricting electron transport while simultaneously monitoring both Δp and respiration (figure 3c) (74, 373). I am aware of only a single study that has applied this approach to β-cell-derived mitochondria, as part of an extensive metabolic control analysis comparing mitochondria prepared from rat skeletal muscle and from INS-1E cells (2). Those from the INS-1E cells respirated slowly (even with succinate as substrate) and maintained a maximal Δp some 40 mV lower than the parallel muscle preparation. Whether this was a consequence of preparative difficulties or reflected a true difference in the properties of the in situ mitochondria was unclear. However, over the range 140-100 mV, the endogenous proton leak was roughly ohmic and exerted a high level of control over respiration and Δp. The authors concluded that extramitochondrial ATP/ADP ratio was controlled much more strongly by the β-cell proton leak than was the case for skeletal muscle mitochondria.

The shape of the current-voltage relationship for the mitochondrial proton leak has important consequences for the ability of substrate availability to modulate Δp. The first detailed current-voltage analyses were performed on isolated brown adipose tissue mitochondria (BATM) (375, 441). In common with β-cell mitochondria, BATM avidly oxidize α-glycerophosphate and possess a substantial endogenous proton leak, additional to their unique uncoupling protein, UCP1 (375). The current-voltage relationship for the endogenous (non-UCP mediated) proton leak of BATM is highly non-ohmic and has been proposed to provide a “safety valve” limiting the maximal Δp attainable in high substrate (441). In the β-cell context, mitochondria operating in a range where the proton leak was severely non-ohmic would show a high flux control over respiration, but little concentration control over Δp, and would therefore be unable to regulate ΔGp by substrate availability. It would therefore be predicted either that the current-voltage characteristics of the leak would differ from that in brown fat mitochondria, or that, even in the presence of optimal substrate, the β-cell mitochondria never approach the range of Δp at which non-ohmic behavior becomes dominant. Affourtit and Brand (2) favor the latter explanation, but the uncertainties regarding the precise quantitation of the maximal in situ Δp and ΔGp maintained by β-cells, which will be discussed below, leave the question open.

Indeed, a near-ohmic relationship is found in the isolated β-cell mitochondrial study of Affourtit and Brand (2) (figure 3c) and is also indicated by the parallel increase in respiration, oligomycin-insensitive proton leak, and Δψm seen in INS-1 832/13 cells during the transition from low to high glucose (figure 3d) (331). Importantly, elevated glucose also enhances ATP turnover, although, as we shall discuss later, there is little or no quantitative information on the ATP utilizing processes in the β-cell.

H. The Contentious Role of UCP2

The mammalian genome encodes at least 50 inner mitochondrial membrane transporters sharing a common 6-transmembrane structure (20). The catalytic functions of many of these have been elucidated, shuttling substrates and metabolites between the matrix and cytosolic compartments. One of these, uncoupling protein 1, UCP1 (SLC25A7), is expressed in brown adipose tissue and functions as a regulated proton leak, activated by free fatty
acids, allowing respiration, and hence thermogenesis, to be liberated from restrictions imposed by ATP turnover (379). In 1997, Fleury et al. (155) identified a human gene encoding a sequence with 59% identity to human UCP1, which they termed UCP2. Of course, simply naming a gene product as an uncoupling protein does not identify its physiological function, and controversy concerning the role of UCP 2 continues to the present day (57, 60, 76, 186, 367, 379).

The initial report that UCP2 mapped close to a diabetes-related locus (155) focused attention on the possible involvement of the protein in insulin secretion and diabetes. Adenoviral overexpression of human UCP2 in rat islets or INS-1 cells increased state 4 respiration (227), decreased GSIS (82), abolished glucose-induced Δψm hyperpolarization detected with R123 (80), and decreased whole-islet ATP content by 50% (80). However, the very extent of these effects raised the suspicion that uncontrolled overexpression of the inner membrane protein was causing non-selective damage to the bioenergetics of the in situ mitochondria. An influential paper by Zhang et al. (551) compared control and global Ucp2−/− deficient mice on a C57BL/6J and 129 mixed background. The knockout animals displayed enhanced fed and fasting plasma insulin and glucose tolerance, while their islets showed considerably enhanced GSIS. The model proposed that a UCP2-mediated proton conductance in control β-cells was sufficient to blunt the mitochondrial hyperpolarizing response to glucose, reducing the elevation in ATP/ADP ratio and restricting GSIS, and furthermore that these effects were amplified in obesity models.

While the concept of UCP2 modulation of the β-cell proton leak has been extensively developed in subsequent years (for reviews, see Refs. 3, 34, 81, 298, 302), doubt was thrown on the interpretation of the original study when the Ucp2−/− mice were extensively back-crossed onto one of three inbred strains (412). In each case UCP2 deletion decreased GSIS, contrary to the earlier observation. Similarly, Parker et al. (403) showed that UCP2 knockout did not improve glucose tolerance in the mouse.

UCP1, even in the presence of the concentrations of fatty acids achieved during active lipolysis, has low specific catalytic activity, and massive amounts of transporter are required, up to 22 μg/mg mitochondrial protein (374, 497), comparable to that of the ANT, to induce a proton conductance in BATM just sufficient to overcome the normal control of respiration by ATP demand (372). While UCP2 has a wide tissue distribution (60), it is expressed at very low levels, comparable to that of most metabolite transporters. The UCP2 content of mitochondria isolated from INS-1E cells depended on the prior nutritional status of the cells, and varied from 2.7 ng/mg mitochondrial protein in the absence of both fetal calf serum and glutamine to 14.5 ng/mg mitochondrial protein in full culture medium containing 20 mM glucose, responding with a half-life of ~1 h (31, 63). Even this latter figure is <0.1% of the content of UCP1 in cold-adapted brown fat. If the assumption is made that UCP2 possesses similar control mechanisms and protonophoric activity to the canonical UCP1, then its contribution to mitochondrial proton leak would be undetectably small, particular in light of the high endogenous proton conductance in these cells discussed above.

In brown adipose tissue, there is a general consensus that UCP1 is activated acutely by the cytosolic unbound fatty acids liberated by β1-adrenergic lipolysis, while its protonophoric activity is terminated at the conclusion of lipolysis by dissociation of the fatty acid from the UCP as the high affinity acyl-CoA synthase depletes the residual fatty acids (301). UCP1 was originally identified by its high-affinity purine nucleotide binding site (214), which, in the cell is considered to be permanently occupied (383). In the artificial absence of nucleotide, UCP1 adopts a nonphysiological high-conductance state (374). UCP2 has been reported to show a detectable proton conductance in the absence of nucleotide that was blocked by GDP (141). The problem is that fatty acids do not appear to activate the nucleotide-bound UCP2 (379), while the physiological significance of a protonophoric activation of UCP2 by high concentrations of superoxide, generated by xanthine/xanthine oxidase or lipid peroxidation products (59), has been questioned by a number of groups (367, 379). In summary, no convincing mechanism to activate a dormant UCP2 has been advanced, in the β-cell context or generally.

Galetti et al. (165) induced an 11-fold overexpression of UCP2 in INS-1 cells, and a similar level of expression of UCP1 in a parallel preparation. Relative to control cells, no effect of these expression levels was observed on insulin secretion or ATP/ADP ratios. UCP1 requires fatty acids for activation, and addition of oleate to the UCP1 expressing cells lowered Δψm and enhanced respiration, consistent with the behavior of the endogenous brown adipose tissue protein. In contrast, the UCP2 expressing cells showed no respiratory or membrane potential responsiveness to the same concentration of fatty acid. The authors concluded that overexpression of the uncoupling proteins was without effect in the absence of an activator, and that only UCP1 could be activated by exogenous fatty acids.

Despite the above, evidence in favor of a classical protonophoric role for UCP2 has been advanced. Affourtit and co-workers (4, 6) compared the oligomycin-insensitive mitochondrial proton leak of control INS-1E cells, cultured in the presence of glutamine to maintain UCP2 protein levels (31, 455), with cells where 80–90% knockdown of UCP2 was achieved by siRNA transfection. The authors observed a decrease in oligomycin insensitive respiration from 13.5 ± 2 to 8.1 ± 1 nmol O·min⁻¹·10⁶ cells⁻¹ associated with the
The terms reactive oxygen species and oxidative stress tend to be ill defined in the literature (365), and most ROS assays are imperfect. With these provisos, a substantial β-cell literature has associated UCP2 with the amelioration of oxidative stress (reviewed in Refs. 6, 93, 413, 445). The concept of “mild uncoupling” as a means of reducing ROS generation (reviewed in Refs. 59, 245) originated with a study in which superoxide generation by isolated mitochondria was greatly enhanced by the supraphysiological Δp achieved with succinate as substrate, but could be reduced by the partial depolarization associated with mild uncoupling (267). However, under more physiologically relevant incubation conditions, superoxide generation is reduced 50-fold (429), and the loss of ATP generating capacity resulting from increased proton leak outweighs any slight effect on ROS (245). The origin, and relation to UCP2, of β-cell reactive oxygen species requires further investigation.

I. Importance of Mitochondrial Content and Morphology

One aspect of β-cell function that is not usually addressed is the critical importance of mitochondrial expression, morphology, and quality control in tuning GSIS sensitivity. A suboptimal mitochondrial content could result in a decreased rate of ATP synthesis, and hence lowered ΔGₚ, at a given glucose concentration, with a resulting decrease in insulin secretion. This is illustrated by a study investigating mitochondrial transcription factor B1 (TFB1M). TFB1M is probably misnamed, since it functions as a specific adenine methyltransferase required for function of the mitochondrial 12S rRNA. In its absence, mitochondrial translation is abolished and the mutation is embryonic lethal (351). Koec et al. (266) identified a common variant in the human TFB1M gene associated with decreased insulin secretion. Islets from these subjects showed dramatic reductions in the levels of mitochondrially encoded complex I subunits. Islets from heterozygous Tfb1m⁺/⁻ mice were exploited as a model of the variant and showed a 50% decrease in complex I ND5 protein level and reduced GSIS. An in vitro model in which Tfb1m expression was reduced INS-1 832/13 cells by siRNA closely reproduced the in vivo data. In a subsequent study (480), mice possessing a β-cell-specific knockout of Tfb1m developed diabetes, while the isolated islets showed profound bioenergetic deficiencies and disturbed mitochondrial morphology.

Whereas electron micrographs give the impression that mitochondria are static within the cell, live-cell fluorescent imaging shows that the organelles are highly dynamic and undergo continuous fusion and fission, and that these processes are critical for mitochondrial quality control and function (295, 296). The mechanisms of fission and fusion are generally conserved between tissues. Fusion is mediated by three GTP-utilizing proteins: Mfn1 and Mfn2 on the outer membrane and Opal on the inner membrane (295). Fission is mediated by Fis1 and Mff on the outer membrane and Drp1, which is recruited to the outer membrane to form a band around the equator of the mitochondrion followed by GTPase-dependent contraction (reviewed in Ref. 295). In both low and high glucose, primary β-cells and INS-1 cells labeled with mitochondrially targeted DsRed reveal a dynamic network of individual mitochondria (356). Activation of a matrix-targeted photoactivated-GFP by a focused laser shows that adjacent mitochondria within the network undergo occasional fusion events, allowing the probe to distribute itself within the two mitochondrial matrices. Fusion events are rapidly followed by fission, generating two daughter mitochondria (521). The concept of quality control originated with the observation that daughters that showed an abnormally low Δψₘ did not reenter the fusion/fission cycle, but were removed by autophagy (521). While lipotoxicity is outside the remit of this review, Las et al. (281) made the important observation that 48-h exposure of INS-1 cells to the supraphysiological unbound fatty acid concentrations frequently employed (~5:1 palmitate to albumin molar ratio, see FIGURE 8) caused fragmentation of the mitochondrial network and defective apoptosis (356).

IV. CENTRAL ROLE OF THE CYTOSOLIC ADENINE NUCLEOTIDE POOL

A. ATP Turnover in the β-Cell

Both oligomycin-sensitive and -insensitive respiration are increased in elevated glucose (FIGURE 3C). While the increased proton current through the leak can be accounted for by the increased Δp, we have virtually no quantitative information on the activity of pathways utilizing cytoplasmic ATP, and how these differ between low and high glucose conditions. In fact, such an analysis would be simple to
perform, by monitoring the acute effects of specific inhibitors on the oligomycin-sensitive component of cell respiration. Birket et al. (50) performed such a study on human embryonic stem cells and derived neural stem cells, and quantified ATP utilization driving protein synthesis, nucleic acid synthesis, proteasome activity, tubulin and actin turnover, and Na⁺ pump activity, by monitoring the acute effects of specific inhibitors on the cells’ oligomycin-sensitive respiration. The proliferating stem cells devoted 33% of their mitochondrial ATP turnover to protein synthesis, as judged by the acute effect of cycloheximide, and it must be born in mind that a study with a dividing insulina cell line might similarly tend to overestimate the ATP demand of protein synthesis relative to that in differentiated primary β-cells.

The continuous turnover of ATP by the β-cell means that a degree of thermodynamic disequilibrium must exist between the proton motive force and the cytoplasmic adenine nucleotide phosphorylation potential $\Delta G_p$. The extent of the disequilibrium will depend on the flux and the control exerted by the ATP synthase and the two inner membrane carriers involved: the adenine nucleotide translocator and the phosphate carrier. Indeed, the study by Affourtit and Brand of mitochondria isolated from INS-1E cells (2) showed that the rate of ATP turnover exerted considerable control over the extramitochondrial ATP/ADP ratio.

B. Some Relevant Equilibrium Thermodynamic Relationships

As in other cells, ATP is continuously turned over in the β-cell cytosol; the steady-state phosphorylation status is thus a dynamic balance between synthesis and hydrolysis. While equilibrium thermodynamics is not applicable under such conditions, it does allow theoretical maximal limits to be set for the Gibbs Free Energy of ATP synthesis achievable under any value of $\Delta p$. Note that while the cytosolic ATP/ADP ratio is usually considered to be the key parameter controlling the open probability of the $K_{ATP}$ channel, the ATP synthesis reaction $\text{ADP + Pi} \rightleftharpoons \text{ATP}$ involves phosphate, which must therefore be included as a variable.

The cytosolic Gibbs Free Energy of ATP synthesis (in kJ/mol), referred to henceforth as the phosphorylation potential $\Delta G_p$, is a function of the displacement of the measured apparent mass action ratio $\Gamma''$ (i.e., [ATP]/[ADP][Pi]), from the apparent equilibrium constant $K'$ obtained under the same conditions of temperature, ionic strength, and $\text{[Mg}^{2+}]$ (382). The free $\text{Mg}^{2+}$ concentration in the mouse β-cell cytosol is close to 0.9 mM regardless of glucose concentration (200), and under conditions relevant to the typical cell cytoplasm ($37^\circ$, pH 7.1, 0.9 mM $\text{Mg}^{2+}$), the apparent equilibrium constant $K'$ for ATP synthesis has been calculated to be $\sim 3.7 \times 10^{-9}$ M⁻¹ (185).

The precise $\text{H}^+/\text{ATP}$ stoichiometry of the mitochondrial ATP synthase is now known from structural analysis of the membrane-located $F_o$ turbine, where the presence of 8 c-subunits suggests that 8 $\text{H}^+$ are required for a 360° rotation and hence synthesis of 3 ATP molecules (529), giving a $\text{H}^+/\text{ATP}$ stoichiometry of 2.67 for the generation of ATP in the mitochondrial matrix. Because of the electrogenic nature of the inner membrane ANT, exchanging ATP$^4^-$ for ADP$^3^-$ (217), the mitochondrial membrane potential drives ATP export and ADP import. Together with the inner membrane phosphate carrier, driven by $\Delta pH$, the overall effect of these carriers is to utilize an additional proton for the export of ATP and import of ADP + $P_i$, (264), making the overall $\text{H}^+/\text{ATP}$ stoichiometry for synthesis and export 3.67.

Armed with this information, it is now possible to calculate limiting values for $\Delta G_p$ that could be maintained at equilibrium in the β-cell cytosol if $\Delta p$ were to vary from 140 to 180 mV depending on glucose availability. The equilibrium relationship is given by

$$\Delta G_p = 3.67F\Delta p$$

where $F$ is the Faraday constant ($0.0965 \text{kJ mol}^{-1} \text{mV}^{-1}$).

FIGURE 5A shows the limiting values for the concentrations of cytosolic adenine nucleotides as a function of $\Delta p$, assuming a total adenine nucleotide pool of 5 mM and a constant $P_i$ concentration of 5 mM. While there are many degrees of uncertainty in these calculations, several points are apparent. First, the ATP concentration is much more responsive to changes in $\Delta p$, while AMP, assuming equilibrium via adenylate kinase, changes by several thousandfold (207). As will be discussed later, the ATP-sensitive K⁺ channel at the plasma membrane might be more appropriately described as an ADP activated channel, the inhibitory ATP binding site(s) being apparently constitutively occupied in the presence of 2–5 mM ATP. At the other extreme, the estimated changes in AMP concentration provide an exquisitely sensitive means of signaling changes in $\Delta G_p$ to the AMP-activated protein kinase, AMPK.

It should be noted that the equilibrium cytosolic ATP/ADP ratio is far higher than that in the matrix, due to phosphate transport and the electrogenic nature of the ANT as discussed above. Thus the extramitochondrial ATP/ADP ratio maintained by isolated rat liver mitochondria could exceed 1,000:1, while that in the matrix was only 9:1 (530). Indeed, as $\Delta p$ drops below 150 mV, the low matrix ATP/ADP ratio maintained by oxidative phosphorylation could predict severe inhibition of matrix ATP-dependent reactions, such as pyruvate carboxylase, a key enzyme implicated in the anaerobic replenishment of mitochondria metabolites in high glucose. However, it is frequently forgotten that one step in the citric acid cycle, succinyl thiokinase, catalyzes substrate level phosphorylation, the GTP formed generat-
ing ATP by transphosphorylation via nucleoside diphosphate kinase. In brown adipose tissue mitochondria, for example, substrate level phosphorylation by 2-oxoglutarate oxidation supported a high \( \Delta G_p \) even when \( \Delta p \) was lowered to 80 mV with a protonophore (381).

## C. Monitoring \( \beta \)-Cell Adenine Nucleotide Pools

The \( \beta \)-cell cytosolic adenine nucleotide pool links the metabolism and bioenergetics of the mitochondrion to the open probability of the plasma membrane K\(_{ATP}\) channel. To integrate the electrophysiological evidence that the cytosolic ATP/ADP ratio controls the open probability of the channel (reviewed in Refs. 11, 133, 422), with the glucose-regulated bioenergetic behavior of the intact \( \beta \)-cell, increasingly sophisticated techniques have been devised, starting with gross ATP determinations on cell or islet populations, then refined to whole-cell ATP/ADP ratios, compartment-selective monitoring of ATP in cell populations, and subsequently at single cell level, and most recently by exploiting probes that appear to monitor cytosolic ATP/ADP ratios directly.

There is considerable debate in the \( \beta \)-cell field concerning the relative importance of the mitochondrion as a bioenergetic regulator of insulin secretion via the cytosolic ATP/ADP ratio, and as a metabolic regulator and source of proposed coupling factors. Resolution of this ambiguity may require more precise quantitative monitoring of the cytosolic adenine nucleotide pool. A candidate that enhances insulin secretion with no discernible alteration in ATP/ADP ratio may legitimately be considered to be a candidate coupling factor. We shall now review the progress that has been made in monitoring the bioenergetic status of the \( \beta \)-cell cytosolic adenine nucleotide pool.

### 1. Whole cell ATP determinations

Simple measurements of acute changes in cell population ATP content in response to glucose are indicative of major excursions in \( \Delta G_p \), but yield little or no quantitative information. There are several reasons for this. First, the \( \beta \)-cell possesses three distinct adenine nucleotide pools: located in the cytosol, the mitochondrial matrix and the relatively unresponsive secretory vesicle pool, each maintaining a distinct phosphorylation potential and responding differently to changes in mitochondrial metabolism. In addition, studies with intact islets have to contend with the heterogeneity of cell type and presence of a hypoxic core. The vesicular pool is particularly problematic. ATP is transported into the vesicles by a nucleotide transporter (170), is co-released with insulin (170), and may provide an autocrine signal acting via P2 receptors to modulate insulin release (65). Most references cite an early study by Detimary et al. (123)
who estimated that some 20–30% of the total adenine nucleotides in rodent islets is located in a metabolically inert granule pool.

The presence of separate cytosolic and mitochondrial adenine nucleotide pools, with very different phosphorylation states, greatly complicates interpretation. Making an arbitrary assumption that 15% of the whole cell adenine nucleotide pool is in the mitochondrial matrix, a drop in whole cell ATP levels resulting from a 20-mV depolarization from 170 to 150 mV could be almost entirely accounted for by changes in the matrix pool, rather than that in the cytosol. Furthermore, such assays do not distinguish between bound and free nucleotide, while comparison of ATP content of two cell preparations cannot distinguish between a thermodynamic difference in $\Delta G_k$ and a simple difference in the total adenine nucleotide pool size. The final problem is that simple ATP determinations are extremely insensitive to the bioenergetic status of the $\beta$-cell, even after all the above problems have been addressed (FIGURE 5A).

2. Whole cell ATP/ADP ratios

Various methodologies have been developed to estimate whole cell ATP/ADP ratios. The approach is, however, still subject to the ambiguities due to compartmental heterogeneity discussed above. ATP has generally been determined in acidic extracts with chemiluminescent luciferase/luciferin (171, 225). The level of ADP in the same extract has been estimated by difference following conversion of ADP to ATP with phosphoenolpyruvate and pyruvate kinase (171, 336). However, this is prone to error at high ATP/ADP ratios, and precision can be improved by hydrolyzing ATP using sulfurylase prior to converting ADP to ATP; this tends to give higher maximal ATP/ADP ratios (112, 157, 388). HPLC detection of adenine nucleotides in cell extracts has occasionally been employed, with the advantage that AMP can additionally be determined, as can nicotinamide nucleotides for monitoring of redox state (334, 405). With these provisos, typical whole $\beta$-cell ATP/ADP ratios increase from ~3 in low glucose to 10–20 on addition of high glucose in isolated $\beta$-cells (387, 506, 509), intact islets (121, 146, 336, 543), INS-1 cells (388, 405), and HIT-T 15 cells (FIGURE 5B) (90). As expected from the previous thermodynamic discussion, most of the change in ratio as glucose is increased would be due to a decrease in ADP rather than an increase in ATP (FIGURE 5B).

D. Compartment-Selective Monitoring of ATP

The exploitation of targeted chemiluminescent or fluorescent probes capable of determining ATP or ATP/ADP ratios has four major advances over whole cell analyses. Errors due to the inclusion of matrix and vesicle pools are avoided; only free, unbound, nucleotides are detected; imagingallows changes in the cytosolic adenine nucleotide pool to be correlated with other parameters; and, finally, when performed at single cell resolution, stochastic events can be detected within the population.

1. Luciferase

Luciferase, derived from the firefly Photinus pyralis, catalyzes the ATP-dependent oxidation of its substrate luciferin in a two-step process that generates photons (334). The chemiluminescent assay may be performed on cell extracts following cell permeation or on intact cells, by targeting the enzyme to specific subcellular compartments. Maechler et al. (325) generated a stable firefly luciferase-expressing INS-1 cell line. Cytosolic ATP was estimated to increase by 16% in the cell population when glucose was raised from 2.8 to 12.8 mM. The technique was extended by Kennedy et al. (258) to target microinjected luciferase constructs to specific subcellular compartments in primary rat $\beta$-cells and MIN6 cells, allowing the responses of single, or small groups, of cells to be monitored. The weak signal required single photon detection, and had limited resolution, but was able to detect a glucose-induced increase in ATP in the matrix, cytosol, and sub-plasma membrane domains, this last determined with a luciferase construct fused to SNAP25, a component of the exocytotic machinery (465). Glucose at 30 mM led to a stable enhancement of ~15% in the chemiluminescence from both the matrix and sub-plasma membrane-targeted constructs, but surprisingly, only a transient increase in cytosolic luciferase signal. While the authors speculated on the existence of a sub-plasma membrane domain preferentially replenished by mitochondrial ATP, the many uncertainties in the luciferase technique suggest caution in this interpretation, particularly since the brief elevation in cytosolic ATP in response to glucose has not been observed with subsequent techniques (506, 510).

2. FRET-based ATP indicators

The group of Imamura and Noji (234, 506) devised a range of Förster resonance energy transfer (FRET) based ATP indicators (“ATEams”) by fusing the NH$_2$ and COOH termini of the $\varepsilon$-subunit of Bacillus subtilis ATP synthase with cyan and yellow fluorescent protein variants, respectively. The constructs could be targeted to the matrix, or, if untargeted, located to the cytosol. In the presence of ATP, conformational changes in the $\varepsilon$-subunit brought the fluorescent proteins closer together, facilitating FRET (234). In studies with INS-1 832/13 cells (183), the affinity for ATP of the AT1.03 ATeam variant was found to be too high to monitor changes in cytosolic ATP in response to glucose or pyruvate; however, the matrix-targeted probe responded sensitively to pyruvate-induced changes in matrix ATP. Addition of 1 mM pyruvate, or glucose to 16 mM increased the matrix ATP concentration 2.5-fold above that recorded for
2.8 mM glucose (183). A more recent ATeam construct, GO-ATeam, with a lower affinity for ATP, has been exploited to monitor both matrix and cytosolic ATP concentrations in both MIN6 insulinoma and mouse islets (506). Consistent with the thermodynamic prediction, the glucose-dependent change in cytosolic ATP was relatively small (FIGURE 5).

3. Fluorescent reporters of ATP/ADP ratios

In 2009, Berg et al. (42) introduced a ratiometric construct (“Perceval”) of a circularly permuted GFP variant (“Venus”) linked to an ATP-binding bacterial regulatory protein, GlkK1. They reported that Perceval responded in vitro to ATP/ADP ratios with a half-maximal response when ATP/ADP was 0.8, and an ability to detect changes in ATP/ADP over a dynamic range from 0.1 to 10. This was not optimal, since cytosolic ATP/ADP ratios, calculated from the phosphocreatine/creatine (PCr/Cr) ratio (see below) may be as high as 200 in some cells. The Perceval signal is also responsive to pH, requiring this to be monitored in parallel to correct the signal. Nevertheless, cytosolic Perceval expressed in mouse β-cells was able to monitor qualitative changes in cytosolic ATP/ADP ratio in response to glucose (511). This study also examined relationships between the ATP/ADP ratio and cytosolic and matrix free calcium concentration, which will be discussed later. Li et al. (293) have questioned the ratiometric basis of the response, by suggesting that while permeabilized, Perceval-transfected, MIN6 cells responded to both ATP and ADP when added separately; the latter signal was due to ATP generated by adenylate kinase. However, their conclusion was based on an effect of the adenylate kinase inhibitor Ap5A added after equilibrium had been attained, and rather suggests a direct effect of the inhibitor on the probe.

Perceval was subsequently modified (“PercevalHR”) to respond to ATP/ADP ratios up to 40:1, with a 3.5-fold dynamic range (507). It should be noted, however, that even this range might be insufficient to accurately record maximal ATP/ADP ratios. PercevalHR binds both ATP and ADP at a single site with high affinity (K<sub>app</sub> 3 and 1 μM, respectively) and the ratiometric response is thus due to competition between ATP and ADP. As a proof of principal, HEK293 cells expressing the Kir6.2 and SUR1 components of the K<sub>A</sub> together with PercevalHR in the presence of 5 mM glucose were exposed to metabolic inhibitors (2-deoxyglucose or iodoacetate) and ATP/ADP ratios were followed while single-channel K<sub>A</sub> currents were measured in cell-attached mode. K<sub>A</sub> channel activity opening probability increased when the indicated ATP/ADP fell below 4 (507).

4. Can the β-cell PCr/Cr pool be used to monitor cytosolic ATP/ADP ratios?

In the myocyte, a highly effective PCr/Cr (phosphocreatine/creatine) shuttle facilitates the rapid and efficient transmission of phosphorylation potential from the mitochondrion to the actomyosin filaments (478). While the skeletal muscle PCr/Cr shuttle is usually described as a means to transport ATP to the myofibrils, its most important role may be to relieve the diffusion limitation for the return of the low micromolar ATP concentration to the mitochondrion and to facilitate a uniform ATP/ADP ratio throughout the cytosol. The mitochondria possess a creatine kinase in their intermembrane space, while a distinct isoform of the kinase is present on the filaments (199). The apparent equilibrium constant for the creatine kinase reaction

$$\text{PCr} + \text{ADP} \rightleftharpoons \text{Cr} + \text{ATP}$$

at 37°C, pH 7.1, and [Mg<sup>2+</sup>] 0.9 mM is 137 (185), and an equilibrium poise in the cell with PCr/Cr ratios close to unity (252) greatly facilitates accurate determinations of high cytosolic ATP/ADP ratios, particularly since PCr and Cr are each present at ~10<sup>-2</sup> M (224). Additionally, since the PCr/Cr pool is purely cytoplasmic and largely unbound, estimations are free from the compartmentalization complications inherent in whole cell adenine nucleotide determinations.

PCr/Cr pools have been detected in insulinoma cell lines (67, 128, 337, 402, 450, 451, 515, 536), as well as primary rodent islets (146, 177, 271). The question is whether a significant PCr/Cr shuttle is operative in β-cells, whether it can be exploited to monitor their cytosolic ATP/ADP ratios, and what the metabolic consequences of such a shuttle might be for adenine nucleotide signaling to the K<sub>A</sub> channel. Ronner et al. (451) used the creatine kinase equilibrium to calculate the free cytosolic ADP concentration in extracts of βHC9 cells as a function of glucose. Phosphocreatine, creatine, and ATP were determined, and with the proviso that not all the assayed ATP would be cytosolic, cytosolic ADP was calculated from the creatine kinase equilibrium. As glucose was increased to 30 mM, ADP fell exponentially to <5 μM, while ATP remained close to 2 mM. A cytosolic ATP/ADP ratio of 400 suggested that the mitochondria in these cells were fully polarized in high glucose, with a Δp greater than 170 mV (FIGURE 5A).

In the previous discussion, it was assumed that the cytosolic P<sub>C</sub> concentration was more or less constant. However, this is not the case in muscle, where hydrolysis of the massive phosphocreatine pool during exercise can elevate P<sub>C</sub> by 500% (268). In elevated glucose, β-cells decrease their matrix and cytosolic free P<sub>C</sub> by 50 and 60%, respectively, as monitored by <sup>31</sup>P NMR (426). The extent to which this is due to sequestration as PCr is unclear; it is notable that a <sup>31</sup>P-NMR spectrum of INS-1 cells published by Wiederkehr et al. (536) shows a glucose-dependent peak in the approximate position expected for PCr. However, net loss from the cell, a so-called “phosphate flush,” has also been reported (158), although it is unclear how the pool would be replenished on the return to low glucose.
Accurate determination of the free cytosolic ATP/ADP ratio is central to the interpretation of metabolic modulations of insulin secretion. It is therefore surprising that such a powerful and quantitative technique as the monitoring of the PCr/Cr ratio has not been more exploited. One potential application, perhaps best performed with a muscle-derived cell line such as C2C12, would be to calibrate the fluorescent response of Perceval or PercevalHR (507) in terms of the absolute ATP/ADP ratios calculated from the PCr/Cr pool as $\Delta p$ is varied, rather than by extrapolation from cell-free determinations (507).

In conclusion, the lack of a “gold-standard” technique to quantify $\Delta p$ has hampered attempts to clearly distinguish between bioenergetic and putative coupling factor mechanisms regulating GSIS.

E. Are There Cytosolic Adenine Nucleotide Micro-Compartments in the $\beta$-Cell?

While there is a temptation to explain discordant observations by invoking micro-compartmentation, it is important to provide some structural or kinetic support for such hypotheses, and the precautionary principle might suggest that experimental limitations first be rigidly excluded. An example in the $\beta$-cell field has been the postulated existence of a sub-plasma membrane environment in which adenine nucleotide concentrations are markedly different from those in the bulk cytosol (e.g., Refs. 258, 386). A localized adenine nucleotide compartment adjacent to the plasma membrane with a diffusion restriction from the bulk cytosolic pool, could only maintain a thermodynamic gradient if ATP was being hydrolyzed (or generated) within that compartment at a rate which exceeded the ability of the nucleotides to equilibrate with the bulk cytosol, in which case the localized ATP/ADP ratio could be lower (or higher) than that in the rest of the cell. To maintain a thermodynamic gradient between two cytosolic locations, it is therefore necessary to demonstrate a combination of high flux and restricted diffusibility. The classic example where this occurs is cytosolic Ca$^{2+}$, whose diffusion is slowed by the presence of low-affinity Ca$^{2+}$ binding proteins, such that waves of elevated [Ca$^{2+}$]$_c$ can be visualized traversing the cell (41), while slow diffusion may allow privileged transfer of the cation between endoplasmic reticulum and adjacent mitochondria (103). It is additionally clear that high, localized Ca$^{2+}$ concentrations from adjacent Ca$^{2+}$ channels trigger synaptic vesicle exocytosis (440) and, probably, insulin granule secretion (86, 462).

We have reviewed the evidence for the presence of creatine kinase, together with a PCr/Cr pool, in $\beta$-cells. The question is whether this is an evolutionary remnant, or whether it plays a significant role in the shuttling of adenine nucleotides from the mitochondria to the plasma membrane, comparable to that in the myocyte. In contrast to that cell, there is little evidence for a localized site of ATP hydrolysis in the $\beta$-cell sufficiently active to create a local zone of ATP depletion, or, alternatively, for mitochondria to selectively deliver ATP to a sub-plasma membrane micro-compartment, where it could be hydrolyzed to ADP before having time to equilibrate with the bulk cytosol. Nevertheless, Schulze et al. (476) speculate that $K_{\text{ATP}}$ channels may be “shielded” from bulk ATP and ADP concentrations by “several phototransfer enzymes [. . . creatine kinase, adenylate kinase, that . . .] together constitute a network that functions as a metabolic barrier.” However, as in muscle, any effect of creatine kinase would be to facilitate, rather than retard, equilibration.

Evidence from targeted reporters is less easy to dismiss. As discussed above, Kennedy et al. (258) targeted luciferase to the mitochondrial matrix, cytosol and sub-plasma membrane (pm) compartments of MIN6 cells (the last by conjugation to the neuronal v-SNARE, SNAP25). In response to 30 mM glucose, chemiluminescence from cytosolic luciferase recorded a transient elevation, returning to baseline within 10 min, whereas pm-luciferase reported a stable elevation. Bearing in mind that ATP (as opposed to ADP or, especially, AMP) is least responsive to changes in $\Delta G_p$, the results were interpreted as indicating that “glucose appears to provoke the formation of a micro-domain of ATP beneath the plasma membrane, which is regulated differently from ATP in the rest of the cytosol” with “preferential delivery of mitochondrial ATP to the sub-plasma membrane domain” (258). However, with the subsequent development of more sensitive fluorescent cytosolic adenine nucleotide probes discussed above, sustained, rather than transient, ATP or ATP/ADP elevations are observed in response to glucose, although oscillations can frequently be detected.

Gribble et al. (191) exploited the ATP sensitivity of a mutant Kir6.2 channel to estimate the sub-plasma membrane ATP concentration, and concluded that there was no gradient of ATP between the bulk cytosol and the sub-membrane domain. Rather than tethering a probe beneath the plasma membrane, Li et al. (293) exploited total internal reflectance fluorescence, TIRF, with Perceval transfected MIN6 cells and adenovirus-infected islets to investigate near-membrane changes in the adenine nucleotide pool. TIRF utilizes an evanescent wave that reaches some 100 nm into a cell from a glass interface under conditions of total internal reflectance, and thus provides a means of selectively imaging relatively near-membrane phenomena, such as vesicle exocytosis (457). It should be noted, however, that TIRF can detect a signal from considerably deeper into the cell than that from a plasma membrane-tethered probe; thus the Kennedy and Li studies are not directly comparable. The latter authors concluded that there was no evidence for a significant gradient of ATP concentration between the TIRF-accessible domain and the bulk cytosol (293).
F. Roles of Adenylate Kinase and AMP

Adenylate kinase catalyzes the reaction
\[ 2\text{ADP} \rightleftharpoons \text{ATP} + \text{AMP} \]

Mammalian cells possess two isoforms: AK1 is cytosolic, while AK2 is located in the mitochondrial intermembrane space. The enzymes operate close to equilibrium in the cell; thus the concentration of AMP in the cytosol may be expressed as a function of that of ATP together with the ATP/ADP ratio
\[ \text{AMP} = \frac{\text{ATP}}{K'(\text{ATP}/\text{ADP})^2} \]

The second power relationship on the bottom line implies that the AMP concentration will change dramatically in response to a change in the ATP/ADP ratio (FIGURE 5A). Since the cytosolic ATP/ADP ratio is proposed to be the key regulatory read-out from the β-cell mitochondria, this implies that the cell possesses two parallel energy-sensing pathways, mediated by the ATP/ADP ratio and the AMP concentration, respectively. Why then has evolution chosen ADP rather than AMP as an “energy sensor” to control the KaTP? The answer might lie in the Goldilocks principle: ATP is too insensitive, AMP too sensitive, but ADP just right.

Depending on the experimental system, three distinct responses to AMP have been discussed in the β-cell context: a direct metabolic effect of the nucleotide, as in other cells, for example in the activation of phosphofructokinase (549); a role comparable to that of the PCr/Cr shuttle in the transmission of phosphorylation potential from mitochondrion to the KaTP (37, 140); and finally, a multiplicity of effects mediated by AMP-activated protein kinase (AMPK) (162).

1. Does adenylate kinase play a direct role in the transmission of ΔGp from mitochondrion to KaTP?

There is some misunderstanding in the β-cell literature concerning the role of adenylate kinase. As extensively reviewed (207), AMP acts as an exquisitely sensitive ΔGp sensor, responding passively to changes in the cytosolic ATP/ADP ratio via the AK equilibrium. However, Dufer et al. (138) have proposed that “activation of AK enhances ADP concentration,” while Beall et al. (37) consider that “AK is the most likely candidate to cause an increase in ADP in the vicinity of the KaTP,” while “inhibition of AK causes a concomitant reduction of ADP levels close to KaTP” and “… altered AK activity … enabling changes in glucose metabolism to be sensed by KaTP channels.” None of these statements is easy to reconcile with the fact that the only significant source of AMP is via AK itself, and that the concentration of AMP is controlled by ATP and ADP, and not vice versa. AK has also been proposed as a component in a “thermodynamically efficient phospho-array that links ATP-generating with ATP-consuming or sensing cellular processes” (140). This proposal is difficult to reconcile with the vanishingly low concentration of AMP under “high-energy” conditions (FIGURE 5A). While 10 mM PCr plus 10 mM Cr can comprise a highly effective transfer system, as in muscle, one relying on 1 μM AMP poses insuperable kinetic difficulties.

2. AMP kinase

AMPK is a trimeric enzyme, consisting of a catalytic α subunit with two regulatory polypeptides, β and γ. The α1 and α2 isoforms of the catalytic subunit of AMPK are expressed in INS-1 and HIT-T15 cells (467, 468) and mouse primary β-cells (116, 162), although the α1 isoform is dominant in cell lines (109) and mouse islets (500). AMPK has more than 30 targets in the cell (207). Generally, the action of the kinase is to inhibit ATP-consuming reactions, such as protein synthesis, and facilitate those associated with ATP generation, either acutely, for example, by activating glycolysis, or chronically by initiating pathways to enhance mitochondrial biogenesis via transcription factor activation. A recent review (162) has evaluated the frequently contradictory literature on the effects of AMPK on GSIS. The authors conclude that, on balance, chronic activation of AMPK reduces glucose-stimulated insulin secretion, while chronic restriction of AMPK activity with dominant negative AMPK has the opposite effect.

Since the focus of this review is the metabolism and bioenergetics of the β-cell, rather than the control of insulin secretion per se, I shall restrict discussion to acute effects modulating the bioenergetic and ion channel responsiveness to altered glucose availability, while acknowledging the evidence for the involvement of AMPK in downstream events including gene expression (106, 109), pre-proinsulin biosynthesis (109), and vesicle dynamics (519). A positive role for AMPK in the acute regulation of insulin secretion is in any case somewhat counterintuitive, since in the presence of high glucose, and hence an elevated ATP/ADP ratio, cytosolic AMP is drastically lowered (FIGURE 5A) and the kinase would be predicted to be relatively inactive. In contrast, a resting β-cell exposed to low glucose concentration would be predicted to have a uniquely elevated cytosolic AMP concentration. Consistent with this, AMPK is active in INS-1 cells in the presence of low glucose, but is rapidly inhibited as glucose is elevated (106, 109, 468).

Two main approaches have been exploited, utilizing respectively genetic modification or supposedly specific activators and inhibitors of AMPK. However, a word of caution is necessary about the latter. Metformin is a drug of choice for the treatment of T2D (119). Its primary target is the liver, where it is accumulated via a selectively expressed transporter OCT1 (organic cation transporter 1) and acutely reduces hepatic glucose production (reviewed in Ref. 526).
However, metformin also has direct effects on the β-cell (275, 276, 280, 336). The current consensus is that metformin, rather than acting as a direct activator of AMPK or its upstream kinase LKB1, functions as a mild, reversible, inhibitor of complex I, and that apparent effects on AMPK are a downstream consequence of restricted mitochondrial activity (35, 69, 73, 145). Furthermore, the ability of the AMP analog and AMPK activator AICAR (5-aminomimidazole-4-carboxamide ribonucleotide) to reduce GSIS from cell lines (468) and islets (106) may require reinterpretation as a result of the finding that effects of the compound on insulin secretion were retained in islets from mice knocked out for both AMPKα isoforms (500). For these reasons, the genetic approach may be considered more discriminating, although faced with the difficulty of distinguishing chronic, adaptive consequences from acute bioenergetic effects.

Islets from mice globally deleted for the AMPKα2 subunit retained normal GSIS in vitro (525). Similarly, islets prepared from trigenic mice with global inactivation of the AMPKα1 gene plus β-cell-specific inactivation of AMPKα2 displayed normal glucose-dependent Δφo depolarization and [Ca2+]e elevation, but significantly enhanced vesicle docking and GSIS, even though the mice were hyperglycemic and insulin deficient (500).

Thiazolidinediones, such as pioglitazone and rosiglitazone, were formerly used in the treatment of T2D. While their chronic effects are mediated by PPAR-γ (411), more direct acute effects on mitochondrial bioenergetics have been proposed. Glucose-stimulated respiration of INS-1 832/13 cells and rat islets was inhibited by pioglitazone (278), and specific mechanisms that have been proposed include inhibition of complex I (73, 167) and an acute inhibition of the mitochondrial pyruvate transporter (125, 342). Each mechanism would have the effect of restricting metabolism and thus indirectly activating AMPK.

The β-cell plasma membrane ATP-sensitive K+ channel, K_ATP, represents the primary point of contact between the upstream metabolic and bioenergetic processes monitoring glucose availability and the downstream generation and regulation of ion channel activity leading ultimately to insulin secretion. The structure and function of the channel has been investigated in exquisite detail over the past 30 yr (reviewed in Refs. 11, 111, 508). It is a 950-kDa assembly of four 6-transmembrane Kir6.2 pore forming subunits as well as four large regulatory SUR1 (ABCC8) sulfonylurea receptor subunits (156). SUR1 is ascribed to the ABCC subclass of ATP binding cassette (ABC) transporters, that include multidrug resistance related proteins and the cystic fibrosis transmembrane conductance regulator (75). Sulfonylurea receptors, however, are distinctive within this class by possessing no known transport activity, while retaining the ability to hydrolyse ATP and undergo consequent conformational changes (11).

The β-cell cytosolic adenine nucleotide pool under differing metabolic conditions, in contrast to the precision with which this parameter has been monitored in cardiac and skeletal muscle. Thus a cytosolic ATP/ADP ratio in excess of 200 can be calculated in resting muscle cells from the PCR/Cr ratio, while even after exercise a cytosolic ATP concentration only ∼20% less than that seen in high glucose (FIGURE 5B).

We have limited information as to the maximum ΔGp attained by β-cells in the presence of excess glucose or alternative substrates. Gerencser (173) reported quantitative ΔGp values of 124–138 mV in low glucose and 152–174 mV in the presence of 16 mM glucose for the mitochondria in dissociated human β-cells from healthy subjects. If the ΔpH values reported by Wiederkehr et al. (536) for the mitochondria in rat β-cells is added to these values, it would suggest that human β-cells can achieve a maximal Δp comparable to that seen in other cells.

An isolated study monitoring the PCR/Cr ratio in creatine-supplemented β-HC9 cells indicated a cytosolic ATP/ADP ratio of ∼200 in high glucose (451). Note that while Perceval-HR is a powerful tool, its signal saturates at an ATP/ADP ratio of 40 (507), so it might fail to accurately report the highest phosphorylation potentials, or help to resolve whether the apparent failure to detect changes in bioenergetic parameters correlating with insulin secretion when glucose is increased above a threshold is due to a limitation in the detection system, or provides evidence for a non-bioenergetic “coupling factor” mechanism.

G. Adenine Nucleotide Interactions With the K_ATP Channel

There remains a lack of rigorous quantitative information on the thermodynamic range of the β-cell cytosolic adenine nucleotide pool under differing metabolic conditions, in contrast to the precision with which this parameter has been monitored in cardiac and skeletal muscle. Thus a cytosolic ATP/ADP ratio in excess of 200 can be calculated in resting muscle cells from the PCR/Cr ratio, while even after exercise to fatigue, the cytosolic ATP/ADP ratio in skeletal muscle is still ∼50:1 (39).

The range and complexity of the electrophysiological literature can be greatly reduced by remembering that, in the intact cell, the cytosolic face of the membrane is continuously exposed to a total adenine nucleotide pool of 5–10 mM in the presence of ∼1 mM free Mg2+. A PCR/Cr ATP regenerating system has the advantage of mitigating the
The regulation of the $K_{\text{ATP}}$ channel is frequently summarized as an inhibitory binding of ATP to Kir6.2 countered by activation induced by Mg.ADP interaction with SUR1. However, electrophysiology reveals a more complex mechanism. The initial condition for most electrophysiological studies is the (nonphysiological) absence of adenine nucleotides, when the channel has a high open probability. $K_{\text{ATP}}$ channel activity on inside-out patches from rat $\beta$-cells was inhibited relative to the nucleotide-free state with a $K_i$ for ATP of 26 $\mu$M in the presence of 2 mM Mg$^{2+}$ (26). A statement that the IC$_{50}$ for ATP in the intact cell is raised to 0.8 mM (508) was based on an early study with intact RINm5F cells where $^{86}$Rb flux was correlated with declining ATP concentrations following metabolic inhibition (471), but before the competing effect of the resulting increase in ADP was recognized. It can therefore be concluded with reasonable certainty that the Kir6.2 site is constitutively occupied by ATP, even in low glucose.

Attempts to investigate the electrophysiology of Kir6.2 in the absence of SUR1 fail because the channel does not insert into the plasma membrane in the absence of the sulfonylurea receptor. However, COOH-terminal truncated forms of Kir6.2 do insert in the plasma membrane of Xenopus oocytes to form functional channels in the absence of SUR1 (520), albeit with decreased affinity for adenine nucleotides, allowing the interaction of adenine nucleotides with the channel itself to be studied. The isolated COOH-terminal truncated form of Kir6.2 displayed an ATP-sensitive conductance with a somewhat reduced affinity ($K_i = 145\ \mu$M) relative to the intact octomer (192, 520). ADP also weakly inhibited the conductance of truncated Kir6.2 (520).

The standard explanation that $K_{\text{ATP}}$ channel opening is regulated by Mg$^{2+}$-dependent ADP binding to the SUR1 subunit is attractive, since on the Goldilocks principle, ATP is too insensitive to changes in phosphorylation potential, while ADP could be “just right.” However, what is required by this mechanism is the demonstration on SUR1 of one or more adenine nucleotide binding sites that demonstrate a sufficiently high selectivity for ADP over ATP to prevent the latter from out-competing the diphosphate. Nucleotide interactions with SUR1 have been investigated by exploiting a mutated Kir6.2 (G334D) that greatly impairs the inhibitory action of ATP on Kir6.2 (423), allowing nucleotide effects selectively mediated at SUR1 to be studied in the intact Kir6.2/SUR1 complex. The intact Kir6.2 (G334D)/SUR1 complex was closed in the nonphysiological absence of adenine nucleotides, but activated by both ADP (EC$_{50}$ 9 $\mu$M) and ATP (EC$_{50}$ 124 $\mu$M) in the presence of Mg$^{2+}$ (111). We are faced with the dilemma that both nucleotides produce effects in the same direction.

An alternative approach has been to express SUR1 in isolation, in which case binding characteristics, conformational changes, and ATP hydrolytic activity can be monitored (111, 115). SUR1 possesses two classes of ATP binding domains: NBD1 and NBD2, the latter showing Mg$^{2+}$-dependent ATPase activity (339). As discussed above, to function as an “ADP sensor,” it might be expected that at least one site would favor the binding of ADP over ATP, at least at ATP/ADP ratios reflecting the low glucose state. Vedovato et al. (523) have advanced an equilibrium, ligand competition, model, in which NBD1 is constituatively occupied by ATP. When NBD2 is unoccupied, Kir6.2 is closed, but opens in response to ATP or ADP binding. Mechanistic discrimination between the two nucleotides would be based on differential dissociation constants for the two nucleotides (18 $\mu$M for ADP and 250 $\mu$M for ATP), a decrease in the ATP/ADP ratio at constant total nucleotide having the effect of slightly shifting the inhibition curve and thus increasing the open probability. ATP hydrolysis at NBD2 is not required in this model.

Alternative models integrate the hydrolytic activity of NBD2 into the mechanism (338, 556). The one depicted in Figure 6A was proposed for the closely related Kir6.2: SUR2A channel. The conformational change in SUR induced by ATP hydrolysis would be transduced to Kir6.2 causing channel opening. ADP and P$_i$ dissociation from NBD2 would revert the conformation to the closed state, completing the cycle. The dissociation of the diphosphate from SUR would be slowed in the presence of increased cytosolic ADP, thus prolonging the open state. Interestingly, Hopkins et al. (228) observed that channel block was enhanced by increasing the total nucleotide concentration at constant ATP/ADP ratio, which would be consistent with the importance of the absolute ADP concentration. Significantly, the cardiac SUR2A possesses a lower affinity for ADP than does SUR1 (338), and much higher ADP concentrations are required to enhance Kir6.2/SUR2A channel opening (340), consistent with activation of the cardiac channel being restricted to conditions of extreme energy depletion (335).

Supporting evidence for a hydrolytic cycle (Figure 6A) has been obtained by the use of inhibitors trapping distinct conformational states of the cardiac Kir6.2/SUR2A...
Beryllium fluoride is a phosphate mimic that, together with ADP, traps the cycle in a prehydrolytic state associated with ATP binding and channel closure. Conversely, orthovanadate stabilizes the posthydrolytic confirmation, leading to channel opening. Both beryllium fluoride and orthovanadate inhibited ATP hydrolysis by a recombinant second nucleotide binding domain (NBD2) of the sulfonylurea receptor, in this case the cardiac SUR2A (556).

Signaling mechanisms tend to have energetic demands; thus information transfer from G protein-coupled receptors requires the hydrolysis of GTP, while ATP utilizing cycles of phosphorylation/dephosphorylation by kinases and phosphatases are almost universal in signaling pathways. The energetic input for conformational changes in voltage-activated channels comes from the movement of charged amino acid residues in response to the altered electrical field across the membrane. The voltage independency of the KATP channel allows individual Kir6.2/SUR1 complexes within a single cell to respond stochastically to changes in the cytosolic adenine nucleotide pool, whereas voltage-gated channels would be entrained by the near instantaneous propagation of a depolarizing wave around the cell, but the question remains as to whether ATP hydrolysis is required to drive a conformational cycle. It could be argued that the ATP/ADP sensor Perceval, discussed previously, is an example of simple ligand competition at the GlnK1 component of the construct. However, it has recently been reported that bacterial GlnK proteins may possess ATPase activity (432). It is therefore entirely reasonable that the precise second-to-second control of the KATP channel should be an ATP utilizing process, driving conformational changes in SUR1 for transmission into the Kir6.2 core. In this sense it may be \( \Delta G_p \), rather than the ATP/ADP ratio, that is the true input to the channel, although I am not aware of any studies in which \( P_i \) concentration has been varied in the presence of a constant ATP/ADP ratio.

Finally, a few caveats. Care should be taken in intact cell studies to control for effects on bioenergetic metabolism by KATP channel effectors. Thus glibenclamide potently inhibits skeletal muscle mitochondrial succinate-dependent state 3 respiration with an EC\(_{50}\) of 1 \( \mu \)M (357), while the related sulfonylurea glyburide potently inhibits state 3 respiration by mitochondria isolated from \( \beta \)-HC9 cells (128). Conversely, the KATP channel opener diazoxide is a weak protonophore and a more potent inhibitor of rat liver mitochondrial state 3 succinate respiration (16, 134, 297), although chronic exposure to 300 \( \mu \)M diazoxide did not appear to affect the respiratory parameters of isolated rat islets (306).

The role, or even existence, of a mitochondrial, diazoxide-sensitive, K\(_{\text{ATP}}\) channel has been debated for many years. Research has primarily focused on its proposed role in car-

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**FIGURE 6.** A: the \( \beta \)-cell K\(_{\text{ATP}}\) channel comprises a Kir6.2 tetramer together with four copies of the SUR1, ABCC-related, subunit. Various models have been proposed to explain the sensitive regulation of channel open probability by \( \Delta G_p \), or the ATP/ADP ratio. The one depicted, based on the cardiac Kir6.2:SUR2A channel, is by Zingman et al. (see also Ref. 556). i: Under physiological conditions, ATP is likely to be constitutively bound to Kir6.2 and perhaps the higher affinity NBD1 site on SUR. ATP binding to NBD2 (ii) and subsequent hydrolysis (iii) creates a conformational change in SUR that is communicated to Kir6.2 leading to channel opening. The cytosolic ADP concentration slows relaxation back into the closed conformation, and thus prolongs the open state. Channel openers also stabilize the post-hydrolytic open conformation. Vanadate traps the cycle in the open, post-hydrolytic, conformation (iii–v), while beryllium fluoride traps ADP and mimics the prehydrolytic ATP-bound closed conformation (Bi). B: channel opening, monitored by cell-attached patch, investigated as a function of ATP/ADP ratio in HEK293 cells stably expressing Kir6.2:SUR1 and transfected with the ATP/ADP sensor PercevalHR (507). The curve was generated by inhibiting glycolysis with iodoacetate, and monitoring channel opening and PercevalHR signal in parallel as \( \Delta G_p \) decayed.
I. Additional Roles of ATP: a Thermodynamic Hierarchy

There is a tendency in the b-cell field to focus on the role of the cytosolic adenine nucleotide pool simply with respect to the modulation of the ATP/ADP ratio and consequent control of the K_{ATP} channel. However, as in any other cell, the pool powers multiple processes required for generic cell functions, as well as the events involved in proinsulin biosynthesis, vesicle trafficking, and the exocytotic process itself. Many of these housekeeping processes must be able to function under both resting, low-glucose, and insulin-secreting, high-glucose conditions. As discussed previously, measurement of the extent to which cytosolic ATP/ADP must decrease to open K_{ATP} channels and inhibit insulin secretion indicates that ΔG_p may be as low as 50 kJ/mol in the resting b-cell, or roughly 10 kJ/mol below that characteristic of a generic cell, such as a myocyte. Put another way, a cytosolic ATP/ADP ratio in the region of 4:1 in a b-cell under low glucose conditions is 50-fold lower than that characteristic of some other cells. While I am aware of no systematic study of relative phosphorylation potential dependencies, the ability of the b-cell to survive and maintain ionic homoeostasis in low glucose media suggests that these vital housekeeping processes remain largely intact. Activation of AMPK under these unique “low ΔG_p” conditions will facilitate cell survival.

1. Metabolism

In terms of metabolism, ATP is required, inter alia, at two steps in glycolysis and for fatty acid activation. As glycolysis responds to an increase in external glucose, one must assume that glucokinase and phosphofructokinase are not limited by cytoplasmic ATP availability in low (2.8 mM) glucose. Furthermore, fatty acid b-oxidation is more rapid in low, rather than high, glucose (332), so acyl-CoA synthase must remain functional under these conditions. In contrast, the phosphorylation potential of the matrix adenine nucleotide pool is always much lower than that in the cytosol, since the latter is boosted by the electrogenic adenine nucleotide translocator (264). Indeed, if we take 140 mM as the minimum Δp in low glucose, this could only support a matrix ATP/ADP ratio of ~0.04 without an input from substrate level phosphorylation (381). As a consequence, the key ATP-requiring anaplerotic enzyme, pyruvate carboxylase, could be severely restricted under these conditions (474).

2. Ion pumps

An occasional event following the addition of high glucose to islets (305, 506) and dissociated cells (179, 221, 434) is a transient decrease in [Ca^{2+}]_c prior to the initiation of oscillations, which has been ascribed to enhanced uptake into the endoplasmic reticulum (ER) (23, 121). This indicates that the ΔG_p maintained in low glucose restricts the activity of one or more pathways sequestering cytosolic Ca^{2+}, such as the plasma membrane and ER Ca^{2+}-ATPases. Thus increasing glucose in the presence of diazoxide, to prevent action potential firing, leads to increased Ca^{2+} uptake into the ER (434). Similarly, there are indications that the kinetics of recovery to basal [Ca^{2+}]_c in single islets is dependent on the cytosolic ATP achieved in elevated glucose (506). In contrast, the Na^{+}-K^{+}-ATPase may have a less stringent ΔG_p requirement, since addition of oligomycin to INS-1 832/13 cells in the presence of pyruvate or elevated glucose leads to rapid and complete repolarization of the plasma membrane as ΔG_p falls and the K_{ATP} channel reactivates (183). This repolarization seems to be maintained, suggesting that the lowered ΔG_p does not restrict the activity of the Na^{+}-K^{+}-ATPase sufficiently to compromise ΔG_p, even with pyruvate as substrate in the apparent absence of an obvious alternative source of ATP.

3. Exocytosis

The ability of glucose to enhance insulin secretion following KCl depolarization in the presence of diazoxide (172) is indicative of a relatively stringent ΔG_p requirement for processes proximal to vesicle trafficking and exocytosis. The exocytotic process itself can be investigated by monitoring plasma membrane capacitance changes, indicative of vesicle fusion, of b-cells exposed to trains of square wave depolarization (reviewed in Ref. 251). By dialyzing the cell with varying concentrations of ATP and ADP, the influence of ΔG_p can be determined (32). Exocytosis in the presence of 3 mM ATP was strongly inhibited in the presence of equimolar ADP within the cell, inhibition being complete within ~4 s (32, 33). Since the capacitance change initiated by the first square-wave depolarization was not affected, this was interpreted as indicating that ADP did not inhibit release of predocked vesicles, but strongly inhibited the priming or translocation of subsequent vesicles for release (32) (see FIGURE 2). The simplest explanation is that exocytosis of docked, but not primed, vesicles has an ATP-dependent step that requires a ΔG_p greater than that achieved in the presence of an ATP/ADP ratio of ~1. An alternative explanation, that granule priming, by acidification of the lumen, is required immediately prior to exocytosis (32) is difficult to reconcile with the instantaneous inhibition of exocytosis on addition of ADP (32), and the observation using luminal pH reporters that the general vesicle population is constitutively acidified (394).

4. Insulin biosynthesis

To maintain the insulin content within the b-cell, secretion must be balanced by subsequent proinsulin biosynthesis.
Protein synthesis is ATP demanding: formation of each peptide bond turns over four ATP molecules, two as a result of aminoacyl tRNA synthetase consuming ATP and releasing AMP and a further two to rephosphorylate the GTP hydrolyzed to GDP by the ribosome during translation. Eight essential amino acids must be imported into the cell, but it is unclear which, if any, of the remaining amino acids are synthesized by the β-cell itself, rather than being imported. The ATP demand for protein synthesis can be roughly estimated from the acute decrease in oligomycin-sensitive respiration of a cell following inhibition of protein synthesis, for example, by cycloheximide (50), but such an analysis does not appear to have been performed for the β-cell.

How do increased glucose availability and/or insulin secretion signal to the protein synthesis machinery? It is debatable whether insulin itself can act in a feed-forward autocrine loop (287). Bearing in mind that the unstimulated β-cell maintains an unusually low ΔGₚ, could protein synthesis be directly controlled by the phosphorylation potential? Leibowitz et al. (286) observed that the cell-permeant monomethyl succinate produced a hyperpolarization of the in situ rat islet mitochondria, and enhanced insulin secretion, to an extent comparable to that with elevated glucose. [³H]leucine incorporation into proinsulin was enhanced by both high glucose and monomethyl succinate, and the authors proposed a direct bioenergetic role for succinate, with a raised ΔGₚ as a proximal signal for proinsulin biosynthesis. Alarcon et al. (14) observed the same potentiation of proinsulin biosynthesis in rat islets in the presence of mono- methyl succinate, but favored an indirect mechanism whereby cytosolic succinyl CoA acted as a coupling factor for biosynthesis. Hypotheses as to the coupling role of acyl CoA esters will be discussed later.

It is perhaps more appropriate to consider how low glucose might restrict, rather than high glucose potentiate, protein synthesis. Since the most distinctive feature of the β-cell is the low ΔGₚ, and hence elevated AMP, in basal glucose, AMP-dependent protein kinase (AMPK) is an established inhibitor of protein synthesis (reviewed in Ref. 207), and is, as expected, active under low glucose conditions (109). A ΔGₚ-mediated activation of proinsulin biosynthesis may thus be mediated by AMPK inhibition, rather than a direct effect of the ATP/ADP ratio (162).

**J. Acute Bioenergetic Aspects of cAMP and Incretins**

cAMP exerts a major influence over insulin secretion. Thus forskolin (232) and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) (87) each enhances insulin secretion from rat islets. Gut-derived incretins, such as glucagon-like peptide 1 (GLP-1), have excited great interest due to their ability to enhance insulin secretion and lower blood glucose (reviewed in Ref. 130). GLP-1 binds to a 7-transmembrane receptor on the β-cell plasma membrane, and its actions are mediated via Gₛα to adenylyl cyclase and cAMP. While 10⁻⁷ M GLP-1 produces stable elevations in cAMP visualized by a FRET sensor (514), low nanomolar concentrations initiate transient cAMP oscillations that are synchronized in individual cells within an islet (514). The two main cAMP targets are protein kinase A and Epac (exchange protein activated by cAMP). Multiple signal transduction pathways have been implicated downstream of PKA and Epac (reviewed in Refs. 130, 164). Targets of interest for the acute regulation of GSIS include the Kₐₜₕ, glucose-dependent closure of which is facilitated by GLP-1 via PKA-dependent phosphorylation, inhibition of voltage-sensitive Ca²⁺ channels, activation of voltage-sensitive Ca²⁺ channels, mobilization of Ca²⁺ from intracellular stores, and PKA-independent facilitation of exocytosis.

In the bioenergetic context, it is relevant to establish whether direct or indirect (e.g., [Ca²⁺]ₘ, mediated) effects of GLP-1 receptor activation impact on the mitochondrion, in terms of metabolism or phosphorylation potential. While cAMP stimulates lipolysis of endogenous β-cell triglyceride stores (548), the role of the resulting fatty acid and long-chain acyl-CoA in influencing GSIS is controversial and will be reviewed in the context of coupling factors.

Working with MIN6 cells, Tsuboi et al. (518) reported that 10⁻⁷ M GLP-1 enhanced the glucose-mediated acute elevation in [Ca²⁺]ₘ, [Ca²⁺]ₘt and cytosolic and matrix ATP (monitored with targeted luciferase). Effects were retained in the presence of EGTA, implicating GLP-1-mediated Ca²⁺ release from internal stores, and were not mediated by Kₐₜₕ closure, since they could be observed in the presence of diazoxide (518). In a subsequent study, Hodson et al. (225) utilized Perceval and reported that the cytosolic ATP/ADP ratio monitored in dissociated β-cells incubated in 17 mM glucose was further enhanced by 20 nM GLP-1. Forskolin and the phosphodiesterase inhibitor IBMX produced comparable large, reversible increases in the ATP/ADP ratio indicated by Perceval. While the responses were not calibrated, the magnitude of the GLP-1- and cAMP-mediated ATP/ADP responses appeared comparable to those during the 3–17 mM glucose transition. While the Perceval probe is operating close to its maximal range under these conditions (42), these results imply that an increase in cytosolic cAMP has large bioenergetic consequences for the β-cell. GLP-1 produced similar effects in the intact mouse islet in the presence of 17 mM glucose, in this case associated with regular ATP/ADP oscillations.

A persistent GLP-1-mediated elevation in [Ca²⁺]ₘ (225) could be consistent with a contribution from Ca²⁺-dependent mitochondrial activation (225, 539), but not with the observation that the increased Perceval signal slightly preceded a rise in [Ca²⁺]ₘ, indicating that Ca²⁺-mediated metabolic activation was not the initiating factor (225). In any
case, the reported magnitude of the effects would appear to be far greater than could be accounted for by a \([\text{Ca}^{2+}]_m\)-mediated citric acid cycle activation, and a plausible mechanism, verified by independent approaches, would seem to be required. One perplexing set of data in this publication is the luciferase determination of total mouse islet ATP levels. In the absence of GLP-1 the transition from 3 to 17 mM glucose produced a doubling of total ATP. Consensus values for the ratio of total islet ATP/ADP in high glucose cluster around 10:1. It is therefore difficult to understand how 20 nM GLP-1 could cause a reported further doubling in ATP unless the total adenine nucleotide pool size increased substantially (225), and a technical problem with the assay should be considered. It should also be noted that Peyot et al. (409) failed to detect an effect of exendin-4 on islet ATP levels in dispersed mouse or rat islets in response to two glucose concentrations.

Any explanation must, of course, obey thermodynamic constraints. The GLP-1 enhancement of the Perceval signal requires either an upstream activation of the proton circuit, or decreased utilization of the proton current. A detailed multi-parameter dissociated cell study relating GLP-1 and cAMP effects on ATP/ADP ratios, \([\text{Ca}^{2+}]_m\), \([\text{Ca}^{2+}]_c\), respiration, \(\Delta \psi_p\), \(\Delta \psi_m\) (and preferably \(\Delta \text{pH}\)) would help to resolve uncertainties.

V. REDOX CONTROL

A. NADPH/NADP\(^+\) Pools

Cytosolic NADPH can be produced by a number of the proposed coupling cycles that will be reviewed later. While the pentose phosphate pathway (PPP) is the classic means of generating cytosolic NADPH, there has been considerable debate as to the activity and importance of the pathway in \(\beta\)-cells (reviewed in Refs. 308, 404, 49). Comparison of \(^{14}\text{CO}_2\) liberation from [1-\(^{14}\text{C}\)]- and [6-\(^{14}\text{C}\)]glucose indicated that the pathway was responsible for \(\sim 6\%\) of the metabolic flux from 5 mM glucose in dissociated rat \(\beta\)-cells (474). A relatively low activity does not, of course, invalidate a pathway as long as it is sufficient to meet the \(\beta\)-cell's ill-defined NADPH requirements. Spegel et al. (491) provided evidence for its significance in INS-1 832/13 cells: following the addition of high glucose, the NADPH/NADP ratio increased in the cells synchronously with the formation of ribose-5-phosphate. However, one difficulty with an obligatory role for the pentose phosphate pathway in facilitating insulin secretion is that alternative substrates, such as \(\alpha\)-ketoisocaproate or pyruvate (in the case of INS-1-derived cells), are highly effective secretagogues while not providing substrate for the pathway. It would, however, be necessary to exclude the possibility of residual glucose in the cells.

Cells have four nicotinamide nucleotide pools, i.e., NADH/ NAD\(^+\) and NADPH/NADP\(^+\) in both matrix and cytosol. Separate analysis is far from trivial and classically requires quantitation of equilibrium metabolite reactions in each compartment [e.g., the cytosolic NADH/NAD\(^+\) ratio calculated from the lactate/pyruvate ratio assuming equilibrium via lactate dehydrogenase (524) which might be inappropriate for the \(\beta\)-cell with its low expression of the enzyme]. Although the NAD(H) and NADP(H) couples both have the same mid-point potential (\(\sim 320\) mV at pH 7), the matrix and cytosolic pools of NAD(H) are both much more reduced than those of NAD(H) in each compartment. In their classic study, Veech et al. (524) estimated free cytosolic NADPH/NADP\(^+\) ratios of 100:1 to 300:1 in rat liver and heart, contrasting with current estimates of \(\sim 0.02\) for the cytosolic NADH/NAD\(^+\) pool (499). Application of this technique to rat (27) or mouse (215) islets confirmed the extreme oxidation state of the cytosolic NADH/NAD\(^+\) pool and determined cytosolic NADPH/NADP\(^+\) ratios of 16:1 and 27:1 (rat) and 27:1 and 58:1 (mouse) in, respectively, low and high glucose. While the authors state that these results give some support to a coupling factor role for the NADPH/NADP\(^+\) couple, I would draw the opposite conclusion: a twofold change in the reduced-to-oxidized ratio implies only a 9-mV change in redox potential, while the concentration of NADPH would only change by 2% as glucose is raised from 3 to 20 mM.

In contrast to the above technique, which ideally determines compartment-selective free nucleotide ratios, total cellular determinations measure matrix plus cytosol and free plus bound nucleotide, with entirely different results, e.g., NADPH/NADP\(^+\) ratios in the range of 0.5:1 to 1:1 (213, 237, 448). Before accepting these values, it must be established that the quenching and extraction techniques employed were sufficiently rapid to prevent artificial re-equilibration of the redox state.

NADH and NADPH have identical, rather weak, autofluorescence spectra. Increasing glucose enhances whole cell NAD(P)H autofluorescence (FIGURE 4B) (112, 147). Much of the signal is localized to the mitochondria in INS-1 832/13 cells (190), due in part to fluorescence enhancement caused by binding to proteins, including complex I. Since changes in whole cell NAD(P)H fluorescence in response to elevated glucose are rapid, with a \(t_{1/2}\) of \(\sim 20\) s (112), it is reasonable to assume that these predominantly represent matrix changes, rather than a slower cytosolic response to anaplerotic metabolite cycling. Two-photon confocal microscopy has been used to directly distinguish matrix and cytosolic NAD(P)H autofluorescence in flattened islets (404). The mitochondrial signal dominated, both in low and high glucose. Leucine enhanced the mitochondrial fluorescence with no significant effect on the cytosol, while 20 mM glucose roughly doubled the autofluorescence in each compartment. Rocheleau et al. (501) attempted to distin-
guish changes in matrix NADH from total islet NAD(P)H, by simultaneously monitoring lipoamide dehydrogenase (LipDH) flavin autofluorescence. The enzyme is a component of pyruvate and 2-oxoglutarate dehydrogenases, transferring electrons to NAD^+. However, the approach assumes that the enzyme operates close to equilibrium, and that the signal reflects LipDH rather than the redox state of the many other flavoproteins in the mitochondrion. With this proviso, the transition from 2 to 20 mM glucose resulted in a parallel increase in LipDH and whole cell NAD(P)H reduction. While quantitation was not possible, this indicated that increased matrix NADH was a significant component of the whole cell NAD(P)H response to glucose, as would be expected. More recently, the possibility of distinguishing between NADH and NADPH fluorescence on the basis of fluorescence lifetime imaging (FLIM) has been demonstrated (51).

High matrix NADP reduction is maintained by both the NADP-linked isocitrate dehydrogenase and the nicotinamide nucleotide transhydrogenase (NNT) that catalyzes the Δρ-driven transfer of electrons from NADH to NADP^+ in the matrix (464, 483). The level of expression of functional NNT, particularly in C57BL/6J and related mouse strains, correlates with the extent of GSIS (29, 483), which does suggest a key role of the reduction state of the matrix NAD(P)H pool. The importance of matrix NADP-linked isocitrate dehydrogenase (IDH2) in the β-cell is unclear; in INS-1 832/13 cells with functional NNT, IDH2 can be extensively knocked down without affecting insulin secretion (312). Not surprisingly, the profound bioenergetic deficit resulting from simultaneous knock-down of both mitochondrial isoforms (IDH2 and IDH3) blocks GSIS (312).

The overall conclusion is that, while maintenance of a reduced matrix NADP pool is almost certainly important functionally, as in any cell, glucose-dependent changes in the cytosolic NADPH/NADP^+ ratio appear insufficient to support a “coupling” messenger role for the nucleotide.

B. Glutathione Pools

Glutathione, GSH, is synthesized in the cytosol by γ-glutamylcysteine synthetase and glutathione synthetase, but is present in millimolar concentrations in both the cytosol and matrix. The inner membrane glutathione carrier remains unknown, previous reports implicating the dicarboxylate or 2-oxoglutarate carriers having been challenged (53). In the matrix, the oxidized disulfide GSSG is reduced to GSH by NADPH-linked glutathione reductase. GSH, in turn, reduces glutaredoxin in both the cytosol and matrix (244), and reduced glutaredoxin acts in turn as a disulfide reductase.

The presence of two pools complicates interpretation of metabolomic studies monitoring whole cell metabolite levels. Green fluorescent protein has been engineered to function as a thiol redox sensor “roGFP” by incorporating two cysteine residues that can form a dithiol, resulting in a change in ratiometric fluorescent properties, depending on the thiol redox potential in the environment (129, 205). The further conjugation with glutaredoxin 1 creates a more rapidly responding probe, GRX1-roGFP2, which specifically equilibrates with the glutathione pool (198, 361). Targeting the construct to the matrix or cytosol allows the local thiol redox potential to be monitored in real time (198). Increasing glucose produced a reductive shift in matrix thiol redox potential in adenoviral transfectected rat islet clusters (504) or INS1-E cells (300). However, no glucose-dependent change in signal was detected from an untargeted, cytosolic, GRX1-roGFP2 probe (504). The implication is that the matrix NADPH and glutathione pools respond conventionally to changes in substrate supply, but that this is not transmitted into the cytosol within the time courses of these studies. While studies with fluorescent probes tend to benefit from independent verification, this finding does not support a metabolically responsive, coupling factor, role for cytosolic NADPH. This is not to say that cytosolic thiol redox state is irrelevant, indeed silencing the cytosolic glutaredoxin-1 in INS-1 832/13 cells strongly inhibits GSIS (438).

VI. CALCIUM INTERACTIONS WITH ENDOPLASMIC RETICULUM AND MITOCHONDRIA

Mitochondrial Ca^{2+} interactions with β-cell metabolism and secretion have been extensively reviewed (179, 539). The inner membranes of mammalian mitochondria, including those in the β-cell (418), possess a Ca^{2+} uniporter (MCU) (36, 113) together with additional, incompletely characterized, accessory proteins (13, 102); uniporter activity increases as the 2.5 power of the ambient Ca^{2+} concentration (557). At equilibrium, the MCU could maintain a 10^5-fold gradient of the divalent cation in the presence of a 150 mV Δψ_m. This catastrophe is prevented by the opposing action of a Ca^{2+}/Na^+ exchanger NCLX (398), which, linked to a Na^+/H^+ exchanger, drives continuous efflux from the matrix. Activity of the efflux pathway is controlled by the matrix free calcium concentration [Ca^{2+}]_m, which increases with Ca^{2+} load until a calcium phosphate complex starts to form (79), after which [Ca^{2+}]_m is essentially buffered at ~2 μM (557) until vast amounts of Ca^{2+} have been accumulated and the mitochondria undergo the catastrophic “permeability transition” (79). Under these “buffering” conditions, respiring mitochondria can accumulate cytosolic extramitochondrial Ca^{2+} until the free external Ca^{2+} concentration ([Ca^{2+}]) falls to ~0.5 μM. This “set-point” (376) is defined by the [Ca^{2+}] at which the rate of Ca^{2+} uptake by the MCU balances the rate at which Ca^{2+} returns to the cytosol via the Ca^{2+} efflux pathway.
Mitochondria in situ show similar behavior (415, 532) and thus provide a means for the temporary storage of excess Ca^{2+}, releasing the cation to the cytosol when [Ca^{2+}]_c falls below the set-point and influx becomes slower than efflux (376). An early study of isolated mitochondria from transplanted insulinomas (418) showed that their calcium transport properties were broadly similar to those previously described for brain mitochondria (384), including a sodium-dependent efflux pathway, with the ability to buffer extramitochondrial free calcium in the 0.4–0.8 μM range.

If matrix Ca^{2+} loading is insufficient for the formation of the Ca-P_i complex, [Ca^{2+}]_m remains below 2 μM and roughly mirrors that in the cytosol, with a hysteresis resulting from the finite kinetics of the pathways (510). An activation of metabolism in response to an elevation in [Ca^{2+}]_m is a general mitochondrial phenomenon, due to enhancement of the activity of three matrix dehydrogenases, pyruvate dehydrogenase (by activating the phosphatase that dephosphorylates the inactive phosphorylated form of the dehydrogenase), and NAD-linked isocitrate and 2-oxoglutarate dehydrogenases, by decreasing the K_m for their substrates (120). Ca^{2+} activation of mitochondrial metabolism is an established component of the mechanism whereby the muscle cell minimizes the drop in ΔG_p during increased activity (182, 299). In addition, cytosolic, as opposed to matrix, Ca^{2+} activates two metabolite transporters, the Mg-ATP/P_i exchanger and ARALAR, the glutamate/aspartate carrier (397), with the latter being a component of the malate/aspartate shuttle contributing to the reoxidation of cytosolic NADH. α-Glycerophosphate dehydrogenase is a further enzyme activated by cytosolic Ca^{2+} that contributes to cytosolic NADH reoxidation, in this case via the glycerophosphate shuttle. Both shuttles are active in INS-1 832/13 cells (495).

A. [Ca^{2+}]_c and Action Potentials

Cytosolic free Ca^{2+} is controlled by the net transport across three membrane systems bounding the cytosol: the plasma membrane, inner mitochondrial membrane, and ER. Each possesses independent pathways for Ca^{2+} uptake and release. The matrix and ER have finite capacities, and in the resting, polarized, β-cell, a [Ca^{2+}]_c close to 100 nM (180) reflects a dynamic balance between Ca^{2+} extrusion from the cell, predominantly via the plasma membrane Ca^{2+}-ATPase (PMCA), and ill-defined constitutive inward “leak” pathways.

The first [Ca^{2+}]_c response of dissociated β-cells to increasing glucose is often a slight decrease (e.g., Refs. 112, 179), suggesting that the activity of ATP-dependent calcium pumps, either at the plasma membrane or on the ER, was previously restricted by the low ΔG_p. Subsequently, single-cell [Ca^{2+}]_c tends to remain close to basal, despite partial plasma membrane depolarization, an increased Δψ_m, and elevation in cytosolic ATP/ADP ratio, until trains of action potentials are initiated (183, 511). Each depolarizing spike correlates with a transient elevation in [Ca^{2+}]_c, which rapidly returns close to basal during intervening quiescent intervals, indicating that Ca^{2+} is rapidly removed from the cytosol across a combination of the three membranes.

B. ER Response to Action Potentials

The β-cell ER (179) and mitochondria (510) each accumulate Ca^{2+} in response to the elevated [Ca^{2+}]_c generated by action potentials. The β-cell ER is partially Ca^{2+}-depleted in low glucose, as monitored by the ER-lumen targeted construct D4ER (434). In the presence of diazoxide, to maintain Δψ_p polarized and prevent additional Ca^{2+} entry to the cell, elevated glucose caused the ER to fill with Ca^{2+} over a period of 10–15 min, while partial emptying following glucose removal required some 20 min (434). This slow response indicates that, while the ER Ca^{2+}-ATPase does respond to changes in ΔG_p, it is too slow to drive any ER-mediated Ca^{2+} oscillations. However, free ER Ca^{2+} ([Ca^{2+}]_m) fluctuations during the ER membrane action potential burst, shadowing Ca^{2+} uptake limits the amplitude of the [Ca^{2+}]_m oscillations and prolongs their duration so that knockout of the major ER Ca^{2+}-ATPase SERCA3 resulted in [Ca^{2+}]_m oscillations that were enhanced in amplitude but recovered more rapidly (434). As in other cells, ER Ca^{2+} depletion by G_c-coupled agonists, such as acetylcholine, or by thapsigargin results in activation of nonselective store-activated Ca^{2+} entry, SOCE. In the β-cell, SOCE is sufficient, in the presence of elevated glucose, to facilitate Δψ_p depolarization (546) and [Ca^{2+}]_m oscillations (23, 179, 447, 505).

In many cells, a close association is seen between ER and mitochondria, maintained by “mitochondria-associated membranes” (MAMs), that are proposed to create micro-domains of high Ca^{2+}, facilitating direct mitochondrial uptake of Ca^{2+} released from the ER (181). However, while Ca^{2+} released from the ER can be taken up by the mitochondria (510), this is considered unlikely to play a role in the control of Ca^{2+} oscillations in β-cells (179, 463).

C. Mitochondrial Ca^{2+} and the Facilitation of Insulin Secretion

An extensive literature links mitochondrial Ca^{2+} uptake to a facilitation of insulin secretion (179, 193, 257, 284, 538). However, it is unclear from the literature whether the apparent effects of elevated matrix Ca^{2+} on insulin secretion can be ascribed purely to conventional bioenergetic effects, e.g., by enhancing Δψ_m, ΔpH, or ΔG_p as a consequence of the activation of pyruvate, isocitrate, and 2-oxoglutarate dehydrogenases (510), whether mitochondrial Ca^{2+} trans-
port is required for the induction and maintenance of plasma membrane oscillations (13), or whether there are additional, less defined, effects relating to the generation of metabolic coupling factors (427, 537).

1. β-Cell mitochondria take up Ca\(^{2+}\) in response to [Ca\(^{2+}\)\(_{m}\)] elevation

At a basal [Ca\(^{2+}\)] of 100 nM, Ca\(^{2+}\) uniporter activity is very low, and the matrix is largely depleted of Ca\(^{2+}\). However, the 2.5 power relationship (557) means that mitochondria can respond sensitively to transient action potential-mediated [Ca\(^{2+}\)] elevations. Rutter et al. (461) observed glucose-mediated [Ca\(^{2+}\)\(_{m}\)] “spiking” with matrix-targeted aequorin in INS-1 cells. Subsequently, Kennedy et al. (256) observed that insulin secretion from INS-1 cells paralleled glucose-dependent increases in [Ca\(^{2+}\)]\(_{m}\) and [Ca\(^{2+}\)\(_{i}\)] and suggested “a fundamental role for Ca\(^{2+}\) in the adaptation of oxidative metabolism to the generation of metabolic coupling factors and the energy requirements of exocytosis.” Parallel monitoring of [Ca\(^{2+}\)\(_{m}\)] with targeted probes such as 2mt8-ratiometric pericam (154) or red-shifted cameleons (528) indicate that at least a proportion of the raised [Ca\(^{2+}\)\(_{m}\)] in response to elevated glucose is transmitted into the matrix after a lag of a few seconds (179, 528). The limited activity of the Na\(^{+}/Ca\(^{2+}\) efflux pathway (557) means that dynamic [Ca\(^{2+}\)\(_{m}\)] responses are damped relative to [Ca\(^{2+}\)]\(_{i}\) (528). Thus the response to a rapid sequence of [Ca\(^{2+}\)] spikes can be a cumulative increase in matrix free Ca\(^{2+}\) (510, 511, 528).

In muscle physiology, the primary consequence of an elevated matrix Ca\(^{2+}\) during exercise is to facilitate metabolism and hence Δp and ATP synthesis (343, 535). In the β-cell, a prerequisite for an elevated [Ca\(^{2+}\)\(_{m}\)] to generate a sufficient ATP/ADP ratio to inhibit the K\(_{ATP}\) channel and fire action potentials would create a logical problem, since action potentials would be required to create the raised [Ca\(^{2+}\)\(_{m}\)] for transmission into the mitochondria. Indeed, while whole cell NAD(P)H fluorescence is an imperfect monitor of mitochondrial Δp, no difference was detected in autofluorescence of human islets or INS-1E cells in high glucose in the presence and absence of extracellular Ca\(^{2+}\) (112). The conclusion was that elevated [Ca\(^{2+}\)]\(_{m}\) was not implicated in the initial glucose-mediated mitochondrial hyperpolarization, and that the whole cell ATP/ADP ratios maintained by INS-1E cells in high glucose were not significantly affected by the removal of extracellular Ca\(^{2+}\) (112). This is consistent with observations that mitochondrial hyperpolarization (183) and glucose-mediated increase in whole cell ATP/ADP ratios (387) precede any rise in [Ca\(^{2+}\)\(_{m}\)].

Multi-parameter analysis of the mouse β-cell response to elevated glucose showed that inhibition of the K\(_{ATP}\) was synchronous with the rise in cytosolic ATP/ADP ratio, as reported by cytosolic Perceval, and preceded the increase in [Ca\(^{2+}\)\(_{m}\)] which was delayed until the onset of electrical activity (511). The [Ca\(^{2+}\)\(_{m}\)] response, monitored with mitochondrial pericam 2mt8RP (154), occurred subsequently as a slow ramp up to a sustained elevation following the rise in [Ca\(^{2+}\)\(_{c}\)] (510).

2. “Second phase” effects

In the absence of acute effects of [Ca\(^{2+}\)\(_{m}\)] on β-cell metabolism, should the focus be turned to the more chronic “second phase”? Various strategies have been employed to modulate the glucose induced rise in [Ca\(^{2+}\)\(_{m}\)]. Wiederkahr et al. (537) targeted a calcium binding protein, S100G (calbindin D-9k), to the mitochondrial matrix of INS-1E and primary rat islet cells, and reported a decreased transient rise in the [Ca\(^{2+}\)\(_{m}\)] response to glucose. When insulin secretion was monitored in parallel, the second phase of insulin secretion, defined as that occurring between 12 and 24 min after glucose elevation, was severely restricted, although the study can be criticized for the use of doxycycline which can affect mitochondrial metabolism (362). Tanaka et al. (506) preloaded islets with the calcium chelator BAPTA. BAPTA is a tetracarboxylic acid, similar to calcium indicators such as fura-2, but lacking a fluorophore; it is conventionally loaded into cells at far higher concentrations than the indicators, with the goal of increasing cytosolic calcium buffering capacity, and damping out changes in free calcium. Surprisingly, BAPTA abolished both the cytosolic and matrix ATP responses to elevated glucose, although the possibility of nonselective toxicity was not excluded.

If the effect of elevated [Ca\(^{2+}\)\(_{m}\)] in β-cells is similar to that in other cells, i.e., activation of pyruvate dehydrogenase and increased substrate affinity for isocitrate and 2-oxoglutarate dehydrogenase, then citric acid cycle function might be facilitated. In view of the depletion of citric acid cycle intermediates following low glucose starvation (FIGURE 7C), and the apparent importance of anaplerotic refeeding for “second phase” secretion, it is reasonable to assume that a depleted cycle could exert significant flux control over mitochondrial respiration, and hence ATP generation. [Ca\(^{2+}\)\(_{m}\)]-mediated pyruvate dehydrogenase activation and increased substrate affinity for the other dehydrogenases could thus facilitate cycle operation during the refilling phase. Quan et al. (427) provided apparent support for this hypothesis when they observed that basal and glucose-stimulated respiration, and hence ATP generation, were both reduced in INS-1E cells following MCU knock-down. However, a surprising complication was that expression levels of electron transport complexes were severely reduced in the knock-down. If this unexpected effect is validated, it will introduce considerable complications into the interpretation of MCU knock-down studies (13, 511).

While removal of external Ca\(^{2+}\) will prevent [Ca\(^{2+}\)\(_{m}\)] elevation by high glucose, it will also inhibit exocytosis and the associated ATP consuming reactions. However, under these conditions, not only was oligomycin-sensitive respiration
ATP turnover) reduced by 60%, but the time-dependent increase in respiration was abolished (112). From first principles, this could be due to a supply side deficit, due, for example, to the lack of Ca$^{2+}$/H$^{+}$ activation of the citric acid cycle, or alternatively to reduced ATP demand by the loss of Ca$^{2+}$/H$^{+}$-dependent cellular processes associated with signaling and exocytosis. The latter is supported by the observation that the whole cell ATP/ADP ratio (with the limitations discussed above) was initially elevated to a similar extent by glucose in the presence or absence of Ca$^{2+}$ (112). Tarasov et al. (511) infected -cells with lentivirus encoding shRNA against MCU. As expected, the rise in [Ca$^{2+}$]$_{m}$ was severely blunted. At the same time, the delayed, second-phase, increase in cytosolic ATP/ADP (but not the first phase) was abolished. Conversely, partial silencing of the mitochondrial Na$^{+}$/Ca$^{2+}$ exchanger NCLX enhanced the elevation of glucose.

FIGURE 7. A: proposed citric acid cycle-derived coupling factor pathways. 1, Malate/pyruvate cycle; 2, pyruvate/citrate cycle (note that this requires stoichiometric utilization of NADPH and malonyl CoA, for example, in de novo fatty acid synthesis); 3, pyruvate/citrate-isocitrate cycles; 4, glutamate. B: levels of key metabolites in INS-1 B32/13 cells after 1 h in 16.7 mM glucose, relative to 2.8 mM glucose. C: inverse plot emphasizing metabolite depletion in low glucose. Data recalculated from Spegel et al. (489).
in $[\text{Ca}^{2+}]_m$ and advanced the second phase ATP/ADP elevation.

Cytosolic ATP (506) and ATP/ADP ratios (506, 510, 511) show biphasic responses to a step elevation in glucose, an initial rapid response being followed by a slower rise. A similar time course can be seen in the respiratory response of clonal insulinoma cells and human islets to acutely elevated glucose, with an initial doubling of respiration followed by a further increase over the subsequent 50 min (112, 184, 491). Much of the enhanced proton current is directed through the ATP synthase, rather than the endogenous proton leak, judged by the extent of oligomycin inhibition (FIGURE 3D) (112, 147, 184, 427). To date, I am not aware of any detailed quantitative analysis of the ATP-consuming reactions in the β-cell cytosol. Such information, particularly comparing basal, first-phase, and second-phase ATP turnover by identified processes, would provide much valuable information.

3. Does matrix Ca$^{2+}$ increase ΔpH?

The simplest explanation for a facilitation of insulin secretion as a result of matrix Ca$^{2+}$ elevation would be an enhanced ΔpH, as observed in muscle. However, direct evidence is surprisingly lacking. Tarasov et al. (511) found no difference in basal Δψ$\text{m}$, or in mitochondrial hyperpolarization in their MCU knock-down in the first 5 min of high glucose, a period during which “second-phase” effects on ΔG$\text{p}$ had become apparent. Quan et al. (427) reported that MCU knockdown did not affect the ability of glucose to hyperpolarize Δψ$\text{m}$, although interpretation was complicated by the decreased expression of electron transport chain components, and the inappropriate JC-1 as a Δψ$\text{m}$ indicator (see Ref. 288).

Remember, however, that ΔpH must be included to calculate the thermodynamically relevant ΔpH. Akhmedov et al. (12) reported a slow increase in ΔpH in INS-1E cells in high glucose. With the caveats discussed above, the study of Quan et al. (427) indicated a decreased ΔpH in their knock-down, in which case the lack of a “second-phase” increase in ΔG$\text{p}$ could be ascribed to a straightforward restriction in the total ΔpH. The extent to which mitochondrial Ca$^{2+}$ accumulation influences ΔpH depends on the extent to which the parallel uptake of P$_m$, which may be thought of as a H$^+$,H$_2$PO$_4^-$ symport (376, 426), discharges the pH gradient. However, in INS-1 cells, both matrix and cytosolic P$_i$ decrease in high glucose (426). This has been ascribed to a net P$_i$ loss from the cells, although the alternative of incorporation into phosphocreatine should be considered, as in muscle (268). In either case, an increased Ca$^{2+}$ uptake and decreased matrix P$_i$ could account for the enhanced ΔpH. The conclusion, therefore, is that a raised ΔpH, due primarily to the ΔpH component, could play a role in the facilitatory role in GSIS played by mitochondrial Ca$^{2+}$ uptake. However, caution is advised in the interpretation of experiments in which the K$^+$/H$^+$ antiporter ionophore nigericin is used in intact cells to collapse ΔpH (12), since the accompanying K$^+$/H$^+$ exchange at the plasma membrane may affect Δψ$\text{p}$ and cytosolic pH (378).

VII. SUBSTRATE DELIVERY TO THE MITOCHONDRION

This review has so far focused on what might be termed the central core of GSIS: the bioenergetics of the mitochondrion, the adenine nucleotide pool, and its interaction with the K$\text{ATP}$ channel. In this section we focus on three bioenergetic inputs to the β-cell mitochondrion: glucose-derived pyruvate, amino acids, and fatty acids. Proposed roles of metabolites as metabolic coupling factors and the basis of oscillatory mechanisms will be reviewed later.

A. Glycolysis

The low-affinity glucose transporter [GLUT2 in the mouse and GLUT1 in humans (114)] is present in considerable excess (216) and thus exerts negligible flux control over glycolysis. In contrast, initial rates of glucose phosphorylation by glucokinase (hexokinase 4) closely match the overall glycolytic rate (216). Glucokinase displays cooperative kinetics with respect to glucose, is not subject to product inhibition by glucose-6-phosphate (99), and is generally accepted to be the key glucose sensor, exerting high flux control over β-cell glycolysis.

Three isoforms of phosphofructokinase-1 (PFK-1) are expressed in mammals: M (muscle), L (liver), and P (platelets, also termed C). Western blots indicate that INS-1 cells express comparable amounts of the P- and M-forms, while the P-form appears to dominate in rat islets (549). PFK-1 is subject to complex regulation (reviewed in Refs. 21, 348). It is activated by its product, fructose-1,6-bisphosphate; ATP, citrate, and phosphoenolpyruvate are allosteric inhibitors, while AMP and fructose-2,6-bisphosphate (generated and hydrolyzed by the bifunctional PFK-2/fructose-2,6-bisphosphatase) are allosteric activators. At first sight, the regulation by adenine nucleotides and citrate would seem to oppose the physiological function of the β-cell, by allowing unrestricted activity in low glucose, when ΔG$\text{p}$ is depressed and the cytosol is relatively depleted of citrate, but opposing the increased glycolytic activity in high glucose, when ATP is high, AMP is low, and citrate has accumulated in the cytosol (184). However, fructose-2,6-bisphosphate can override the feedback inhibition by ATP and citrate, while PFK-2/fructose-2,6-bisphosphatase may additionally interact with and regulate glucokinase (21). The proposed role of PFK-1 and PFK-2 in the generation of oscillatory glycolytic activity will be reviewed later.
1. “Forbidden” pathways: lactate dehydrogenase and monocarboxylate transport

When a muscle cell experiences a sudden increase in ATP demand that cannot be met by increased oxidative phosphorylation, the fall in $\Delta G_p$, and hence increase in ADP and AMP, removes kinetic restraints on glycolysis, notably at phosphofructokinase, allowing a massive increase in the rate of glycolysis to augment ATP generation. The excess pyruvate, beyond that entering the mitochondrion, is reduced to lactate and exported. In this process the rate of glucose utilization may increase 10-fold or more. This requires lactate dehydrogenase (LDH) and a plasma membrane monocarboxylate transporter (MCT) to allow lactate to be generated and exported from the cell, and, most importantly, a glycolytic pathway capable of achieving the required rates. Under low glucose conditions, the $\beta$-cell, with its high upstream flux control at the level of glucokinase, cannot accelerate, despite the removal of kinetic restraints by the lowered $\Delta G_p$. Far from augmenting the cell’s ATP production, the presence of LDH and a MCT could further restrict ATP generation by diverting the limiting pyruvate supply away from the mitochondrion. The presence of two alternative fates for pyruvate, reduction to lactate and mitochondrial oxidation, would disturb the coupling between glucose and $\Delta G_p$, since the former pathway generates only 2 ATP molecules per glucose, whereas some 15–30 (depending on the coupling efficiency) are produced by oxidative phosphorylation. The absence, or very low expression, of LDH and the MCT in primary $\beta$-cells prevents this from occurring (424, 430, 477, 554). The latter deficiency performs an additional physiological role in preventing circulating pyruvate and lactate produced during exercise from triggering insulin secretion. Together, the absence of these “forbidden pathways” facilitates a stoichiometric coupling between glucose availability and mitochondrial function. INS-1-derived cell lines displaying robust glucose-sensitive insulin secretion have low expression of LDH (17, 477). Thus RINm5F cells show a greatly enhanced lactate production in addition of a mitochondrial inhibitor, while INS-1 cells respond with a further reduction of an already low level (477). It should be noted finally that the list of “forbidden” pathways has been considerably expanded by studies at the transcriptional level (424).

2. Reoxidation of glycolytic NADH

In the absence of lactate dehydrogenase, the NADH generated in the cytosol by glycolysis must be stoichiometrically reoxidized by the mitochondria via either the malate/aspartate or $\alpha$-glycerophosphate shuttles. As in other cells, the thermodynamic problem inherent in transferring electrons from the highly oxidized cytosolic NADH/NAD$^+$ pool to the matrix is resolved in the former case by utilizing the proton circuit to drive the glutamate/aspartate carrier, and for the $\alpha$-glycerophosphate shuttle by feeding the electrons into the UQ pool, bypassing complex I. $\alpha$-Glycerophosphate is an effective substrate for permeabilized $\beta$-cells (307, 477), and inhibition of the malate/aspartate shuttle in intact INS-1 832/13 cells with phenyl succinate did not inhibit the glycolytic response, indicating that the activity of the parallel glycerophosphate shuttle in these cells was sufficient to allow reoxidation of cytosolic NADH (495). Similarly, both shuttles function in parallel in mouse islets (435).

3. Glycolytic metabolite levels during the low to high glucose transition

The relative levels of glycolytic intermediates increase in a time-dependent manner following the low to high glucose transition.
transition in INS-1 832/13 cells and rat islets (489–491). While the precise patterns differ, glucose-6-phosphate, fructose-6-phosphate, glyceraldehyde-3-phosphate, and pyruvate all show a time-dependent, severalfold, increase. Of particular interest, the levels of intracellular pyruvate increase 10-fold following the transition from 2.8 to 17 mM glucose. Pyruvate, of course, is the primary substrate for both mitochondrial oxidative metabolism, via pyruvate dehydrogenase, and the anaplerotic processes, via pyruvate carboxylase, replenishing citric acid cycle intermediates and implicated in enhancing the secretory response to elevated glucose.

As will be discussed more extensively in the context of the citric acid cycle, the convention seems to be that the starting, low glucose, state is considered to provide a baseline, with high glucose enhancing glycolytic intermediate levels. If, however, this is inverted, and metabolites are referred relative to the high glucose state, then 2.8 mM glucose can be considered as a substrate-depleted starvation state, with elevated glucose simply restoring the "normal" metabolite levels (FIGURE 7, B AND C). It may be significant that, at least in some studies (108), biphasic insulin secretion is not seen when islets are preincubated in a more physiologically relevant 5.6 mM glucose.

B. Pyruvate

The expression of the plasma membrane monocarboxylate carrier in most insulinoma cell lines allows exogenous pyruvate to be utilized as a metabolic fuel. In INS-1 832/13 cells, as little as 1 mM pyruvate generates the same bioenergetic effects on respiration, ATP levels, and insulin secretion as 17 mM glucose (183). Pyruvate can simplify experimental design and interpretation by bypassing glycolysis. In primary β-cells that lack the MCT, pyruvate can be presented to the cell as the membrane-permeant methyl ester (107, 506), which is hydrolyzed within the cytosol to yield pyruvate (plus, unfortunately, methanol). Methyl pyruvate is an efficient secretagogue (329), although it has been suggested that it may have nonmetabolic effects on the KATP channel (136).

In contrast to the structurally related six-transmembrane carrier family responsible for the transport of most metabolites across the inner membrane, pyruvate transport is reported to be mediated by a heteromeric association of two novel three-transmembrane proteins termed Mpc1 and Mpc2 (68, 223). Interestingly, pioglitazone, a member of the thiazolidinedione class of antidiabetic agents that had previously been shown to restrict β-cell mitochondrial metabolism (278, 279), was reported to act as a selective inhibitor of the pyruvate transporter (125).

1. Pyruvate dehydrogenase

The pyruvate dehydrogenase complex (PDC) comprises pyruvate dehydrogenase (PDH), dihydrolipoyl transacetylase, and dihydrolipoyl dehydrogenase. PDH is inactivated by phosphorylation by pyruvate dehydrogenase kinase and reactivated by pyruvate dehydrogenase phosphatase (for review, see Ref. 120). Not surprisingly, β-cell specific knock-out of the pyruvate dehydrogenase subunit dramatically inhibits GSIS (492). There are conflicting reports on the ability of PDH to regulate β-cell bioenergetics. Nicholls et al. (385) found no effect of kinase or phosphatase overexpression on GSIS in MIN6 cells, while Krus et al. (273) reported that partial knock-down of PDH kinase-1 in INS-1 832/13 cells enhanced GSIS. Note that significant flux control in the intact β-cell at the level of PDH implies that upstream control at glucokinase must be lowered, for example, during elevated glucose.

2. Pyruvate carboxylase

Pyruvate carboxylase (PC) generates oxaloacetate from pyruvate and HCO₃⁻. The enzyme utilizes matrix ATP and is allosterically activated by AcCoA (242). It is highly expressed in most β-cells (346, 474), although human islets have much lower levels than rodent islets or INS-1 832/13 cells (319). The requirement of the pathway for HCO₃⁻ should be born in mind when employing bicarbonate-free incubation media, for example, in certain Seahorse Respirometer experiments. The enzyme is relatively inactive under low glucose conditions, not only because of the limited availability of pyruvate and its allosteric activator, acetyl CoA, but also because matrix ATP levels are low, bearing in mind that the matrix ΔGₚ is always much lower than that in the cytosol, due to the electrogenic nature of the adenine nucleotide transporter (264). Thus a Δp of 140 mV can only support a matrix ATP/ADP ratio of the order of 0.04.

PC catalyzes a key anaplerotic reaction. The classical citric acid cycle involves a two-carbon input from acetyl CoA, derived from pyruvate or fatty acid, at citrate synthase, followed by the loss of two moles of CO₂ by isocitrate and 2-oxoglutarate dehydrogenases, and thus results in no change in intermediate pool sizes. In contrast, a dual input from pyruvate via PDH and PC, with the formation of acetyl-CoA, and oxaloacetate results in the net synthesis of citrate and downstream metabolites, i.e., anaplerosis. PC is not the only anaplerotic pathway capable of replenishing the citric acid cycle, since, for example, glutamate dehydrogenase and aspartate transaminase can contribute 2-oxoglutarate and oxaloacetate, respectively, to the cycle.

In other tissues, anaplerosis replenishes the citric acid cycle to compensate for metabolites withdrawn from the cycle for biosynthetic purposes such as gluconeogenesis in liver and lipogenesis in adipose tissue, and to contribute to metabolic cycles generating the cytosolic NADH and NADPH re-
required for these pathways (242). However, the \( \beta \)-cell does not perform gluconeogenesis (337) and has a limited capacity for fatty acid synthesis (but see Ref. 317). Why then does PC-mediated anaplerosis appear to occupy such a central position in metabolic investigations into GSIS? Is it simply to refill a depleted citric acid cycle resulting from a low glucose preincubation, or to generate specific metabolic coupling factors required for optimal exocytosis? This will be reviewed later.

C. Amino Acids

While glucose is the dominant physiological regulator of insulin secretion, amino acids such as alanine, leucine, and glutamine can also induce insulin secretion (254, 371). In terms of mechanism, it is important to determine whether their support of insulin secretion correlates with their ability to raise \( \Delta G_m \), or whether metabolic coupling factors have to be considered.

1. Glutamine

The complexity of glutamine and glutamate metabolism in the \( \beta \)-cell is the subject of a number of reviews (149, 161). \( \beta \)-Cells metabolize added glutamine, which can enter the citric acid cycle via glutaminase, transport into the matrix through the glutamate carrier (SLC25A18, 22), and generation of 2-oxoglutarate via glutamate dehydrogenase. Glutamate dehydrogenase is inhibited by GTP and activated by ADP, and thus responds to the energy status of the mitochondrion. Additionally, the enzyme is allosterically activated by leucine, or its nonmetabolizable analog \( \beta \)-2-aminobicyclo[2,2,1]heptane-2-carboxylic acid (BCH), and experimentally glutamine is often added in combination with one of these activators (208, 506). The amino acid can thus contribute to citric acid cycle anaplerosis. It facilitates GSIS but does not induce insulin secretion in low glucose, except from islets prepared from high-fat fed mice (153). The literature is largely focused on the role of glutamine in anaplerosis and the generation of coupling factors rather than as a bioenergetic substrate. However, Tanaka et al. (506) utilized the targeted ATP reporter GO-ATEam and found that while 10 mM glutamine had no effect on mitochondrial or cytosolic ATP levels of islets in the presence of 2.8 mM glucose, the combination of glutamine and leucine caused a rapid increase in ATP in both compartments of a magnitude comparable to that seen with 25 mM glucose. Similarly, Gerencser (173) demonstrated that the combination of glutamine and BCH hyperpolarized \( \Delta \psi_m \) in human islets.

2. A role for sirtuins?

In 2001, the group of Ziegler (222) reported that liver mitochondrial glutamate dehydrogenase could be inhibited, in the absence of \( \text{Mg}^{2+} \), by an endogenous, \( \text{NAD}^{2+} \)-dependent, ADP-ribosylating activity and reactivated by an endogenous ADP-ribosylcysteine hydrolase on \( \text{Mg}^{2+} \) readdition. Strong inhibition of ADP-ribosylation by \( \text{NADH} \) and \( \text{NADPH} \), together with the requirement for \( \text{Mg}^{2+} \) removal, led the authors to conclude that “under normal conditions, ADP-ribosylation of GDH would be expected to be rather low.” Subsequently, the Guarente group (203) detected the sirtuin SIRT4 in mouse \( \beta \)-cells and MIN6 cells and demonstrated a mitochondrial association in the latter, proposing SIRT4 as a negative regulator of amino acid stimulated insulin secretion. Recombinant SIRT4 was able to ADP-ribosylate bovine glutamate dehydrogenase in vitro in the presence of \( \text{NAD}^{+} \), and to inhibit its activity; assay conditions were not given, except with reference to an assay in the (unphysiological) direction of glutamate formation from 2-oxoglutarate plus 200 \( \mu \text{M NADH} \) and 50 mM \( \text{NH}_4^{+} \), in the apparent absence of other activators or inhibitors (222). One complication is that the presence of 1 mM \( \text{NAD}^{+} \), required for ADP-ribosylation, will itself inhibit this GDH assay. The most dramatic claim in the Haigis paper from a bioenergetic standpoint was that SIRT4-RNAi treatment of MIN6 cells doubled respiration in high glucose, as well as doubling ATP levels in both low and high glucose, while enhancing GDH activity by just 20% (see Ref. 203). Leaving aside the limitations of simple ATP assays, as discussed above, it is difficult to envisage a mechanism whereby glucose-mediated bioenergetics could be so dramatically affected by slight changes in the activity of an enzyme peripheral to the canonical GSIS (as opposed to glutamine-stimulated insulin secretion) pathway. In a commentary, Argmann and Auwerx (22) raise additional questions as to the physiological significance of this putative regulatory mechanism.

A second \( \beta \)-cell sirtuin, the nuclear-located SIRT1, has been implicated by the same group in the control of insulin secretion, this time as a positive modifier (54). Global-SIRT1 KO mice failed to secrete insulin in response to glucose and showed impaired glucose tolerance. Similarly, islets from the mice failed to demonstrate GSIS, as did both INS-1 and MIN cells treated with SiRNA to SIRT1. In a search for a mechanism underlying this dramatic failure of GSIS, the authors focused on UCP2. As previously discussed, I share a certain skepticism concerning the ability of trace amounts of UCP2 to influence mitochondrial proton conductance and negatively impact on GSIS. Nevertheless, Bordone et al. (54) proposed that UCP2 is upregulated in SIRT1 knockout mice and knockdown cells sufficiently to increase the endogenous proton leak and abolish GSIS. A modest increase in UCP2 was indicated in Western blots of both INS-1 knockdown and knockout mice, but this was not quantified, proton leak was not determined directly, and some of these data were later retracted (55).

A comprehensive critique of the sirtuin field is out of the scope of this review. Nevertheless, in view of the enormous
interest in this area (the two papers discussed above have been together cited almost 800 times), it is essential that claims relating to metabolism and bioenergetics be evaluated with the same rigor required of other aspects of the \( \beta \)-cell field.

3. Arginine and Leucine

The cationic amino acid arginine is not strictly a metabolite, but high concentrations of \( l \)-arginine, and certain nonmetabolizable arginine analogs, potentiate glucose-mediated \( \Delta \psi_p \) depolarization as a direct consequence of their electronegenic uptake through the mCAT2A carrier (352, 479, 486, 512). In contrast, leucine is the only physiological amino acid capable of stimulating insulin secretion in the presence of low glucose by metabolic means (149). The amino acid can be trans-deaminated to \( \alpha \)-ketoisocaprate (\( \alpha KIC \)) which can ultimately be metabolized to AcCoA and acetoacetate and is thus a potential bioenergetic substrate, in addition to ability to activate glutamate dehydrogenase. \( \alpha KIC \) is an effective secretagogue in combination with methyl succinate (318, 321), although it has been reported to be less effective than glucose in increasing NAD(P)H reduction or hyperpolarizing the mitochondria (400).

D. Synthetic Substrates: Methyl Esters

The impermeability of the \( \beta \)-cell plasma membrane to many citric acid cycle intermediates has prompted the use of permeant synthetic methyl esters as artificial means of supplying substrate to the mitochondria. Nonselective esterases in the cytosol regenerate the native metabolite (together with methanol). Mono- and dimethyl succinate as well as methyl pyruvate have been most commonly employed. Methyl pyruvate is a secretagogue for primary \( \beta \)-cells, able to bypass the lack of a monocarboxylate carrier (329). It has, however, been suggested that the ester has additional effect beyond the generation of cytosolic pyruvate (136, 291). Methyly-2-oxoglutarate is a very potent secretagogue in mouse islets (431), while succinate esters are effective secretagogues in INS cells and rat (but not mouse) islets, hyperpolarizing the mitochondria, increasing NAD(P)H reduction, and depolarizing \( \Delta \psi_p \) (213, 273). The ineffectiveness in the mouse has been ascribed to the lack of malic enzyme, preventing cytosolic succinate generated in this way from being anaplerotic, since the dicarboxylate carrier would exchange succinate for malate, with no net increase in citric acid cycle intermediates (400). However, the carrier exchanges succinate or malate for \( P_i \) (397), and at equilibrium each dicarboxylate will distribute across the inner mitochondrial membrane as a function of the square of the proton gradient (444). Thus, in an intact cell, succinate esters should increase the citric acid cycle pool. As would be expected, the complex II inhibitor 3-nitropropionate has severe bioenergetic consequences (142).

The absence of precise quantitative determinations of \( \Delta \psi \) and \( \Delta G_p \) in intact \( \beta \)-cells means that the extent to which insulin secretion evoked by these alternative substrates is predominantly controlled by bioenergetic factors, as suggested by Antinoozi et al. (19), rather than metabolic coupling factors, remains undecided.

E. Fatty Acids

In muscle, liver, and adipose tissue, glucose and fatty acid compete for oxidation (reviewed in Ref. 231). In the fasting state, \( \Delta G_p \) is maintained by fatty acid oxidation and glucose is spared, most potently by inhibition of pyruvate dehydrogenase by a combination of raised AcCoA and NADH and activation of the inhibitory pyruvate dehydrogenase kinase (493). \( \beta \)-Cells oxidize exogenous fatty acids provided as albumin buffers with typical, physiologically relevant, fatty acid/albumin molar ratios not exceeding 3:1 (127, 279, 407, 446). The cells also possess endogenous triglyceride stores (328) together with low levels of hormone-sensitive lipase activity (363, 545). Lipolysis, monitored by glycerol release, increased with glucose concentration and correspondingly paralleled insulin secretion (545), while AMP elevation by forskolin or the incretin GLP-1 enhanced lipolysis and \( \beta \)-oxidation in, for example, HIT-T15 cells (547). Although lipolysis correlates with insulin secretion, \( \beta \)-oxidation shows an inverse correlation. As in other cell types, \( \beta \)-oxidation is inhibited in high glucose, via activation of Ac-CoA carboxylase, generation of malonyl-CoA and consequent inhibition of carnitine-palmitoyl transferase-1 (CPT-1) (85, 332, 407, 456).

Paradoxically, from a bioenergetic standpoint, fatty acids are ineffective as secretagogues in low glucose when they can be oxidized (e.g., Ref. 189), but potentiate glucose-stimulated secretion when \( \beta \)-oxidation is inhibited (201, 420). This has led to a focus on nonbioenergetic mechanisms of facilitation, which will be discussed later. From a bioenergetic standpoint, however, it is important to establish whether acutely administered fatty acids fail to evoke secretion in the presence of low glucose simply because they fail to elevate \( \Delta \psi \) and \( \Delta G_p \) sufficiently to close the \( K_{ATP} \), or whether additional constraints are present.

In other tissues, \( \beta \)-oxidation would first be characterized using isolated mitochondria, with the addition of either acyl carnitines or free fatty acids in the presence of CoA, ATP, and carnitine (e.g., Ref. 383). The paucity of respiratory studies on isolated \( \beta \)-cell mitochondria or permeabilized cell preparations, perhaps exacerbated by the apparent difficulty of such preparations to utilize complex I, has meant that such studies are virtually nonexistent, and it is surprisingly difficult to locate any publications employing isolated mitochondria, or permeabilized \( \beta \)-cells, exposed to these substrates. One exception was the use of luciferase to detect ATP generation by a heterogeneous preparation of mito-
chondria isolated by centrifugation from ob/ob mouse islets (233, 289). Succinate, glycerol-3-phosphate, pyruvate plus malate, and palmitoyl carnitine were each reported to be substrates, although the limitations of the technique precluded any further investigation. Civelek et al. (91) permeabilized HIT insulinoma cells with saponin and compared rates of \(^{14}\)CO\(_2\) production from \([^{14}\text{C}]\text{pyruvate}\) and \([^{14}\text{C}]\text{palmitate}\). The latter rate was minuscule: 15 fmol·min\(^{-1}\)·mg protein\(^{-1}\) compared with 1 nmol·min\(^{-1}\)·mg protein\(^{-1}\) for pyruvate, although in the apparent absence of added CoA or carnitine it is difficult to see how the fatty acids could be activated.

The significance of fatty acid oxidation for the intact \(\beta\)-cell can be assessed by monitoring the extent of \(\beta\)-oxidation of endogenous fatty acids, or of exogenous fatty acid/albumin buffers. However, a recurring concern in a survey of the \(\beta\)-cell fatty acid literature is the use of supraphysiological free fatty acid concentrations. Circulating fatty acids are bound to albumin, with typical molar ratios in the range 1:2 for the human (442) and up to 3 for the fasted rat (496). The free, unbound, fatty acid concentration under these conditions is only \(-10-20\) nM (105, 442). While the need for albumin binding is generally appreciated in the literature, molar ratios of 6:1 or even higher have been employed in vitro (144, 285, 332, 472), producing unbound fatty acid concentrations close to 1 \(\mu\)M, or 100 times higher than the normal physiological range (FIGURE 8A). One concern in such studies is the possibility that excessive fatty acid exposure in vitro may produce artifactual changes in metabolism, for example, due to nonspecific mitochondrial uncoupling (269, 277), that might mask a physiologically relevant positive signaling or bioenergetic effect.

Some of the earliest determinations of endogenous fatty acid oxidation (328, 330) monitored \(^{14}\)CO\(_2\) evolution from islets prelabeled with \([^{14}\text{C}]\text{palmitate}\). Assuming complete oxidation by the citric acid cycle, the authors concluded that \([^{14}\text{C}]\text{palmitate}\) oxidation could account for only 0.2% of basal respiration (328), but were unhappy with the technique due to difficulties in assessing equilibration with different pools, and instead estimated rates of fatty acid oxidation comparable to liver based on the small fall in triglyceride content. This rather unsatisfactory technique seems to be the basis for a number of subsequent statements that endogenous fatty acids are a primary fuel for unstimulated \(\beta\)-cells (e.g., Refs. 91, 328). An alternative approach is to inhibit \(\beta\)-oxidation at carnitine-palmitoyl transferase-1 (CPT-1) by etomoxir (85) or methyl palmoxirate (330). If \(\beta\)-oxidation provided a glucose-sparing function in low glucose similar to that in other tissues, then it might be expected that inhibition of CPT-1 would increase glucose utilization. However, these inhibitors had no effect on the utilization of glucose or glutamine (330).

INS-1-derived cells appear not to share the problems of exogenous fatty acid incorporation into oxidizable pools. In low glucose, INS-1 832/13 cells oxidized labeled palmitate to \(^{15}\)CO\(_2\) at rates of 0.2–0.4 nmol·min\(^{-1}\)·mg protein\(^{-1}\) (279, 332, 407). These rates are comparable to those for the utilization of 2.8 mM glucose by these cells (332). It should be noted that INS-1 832/13 cells can produce acetoacetyl-CoA and utilize \(\beta\)-hydroxybutyrate and acetoacetate (318, 320), raising the possibility that ketone bodies could be produced by \(\beta\)-oxidation, in which case label from \(^{14}\text{C}\)-labeled palmitate would not be liberated stoichiometrically as \(^{14}\text{CO}_2\).

It is clearly important to determine the bioenergetic consequences of fatty acid oxidation and the interplay with glycolysis, particularly in low glucose. If, as is generally believed, fatty acids primarily perform a coupling rather than energetic role, is their contribution to basal metabolism merely insufficient to raise \(\Delta G_p\) to levels closing the \(K_{\text{ATP}}\), or are there other factors in play, such as the inability of fatty acids to play an anaplerotic role in the citric acid cycle (548)? El-Azzouny et al. (144) performed a wide-ranging analysis of the metabolic consequences of exposing INS-1 832/13 cells to palmitate/albumin buffers in the absence and presence of high glucose. With the proviso that the authors employed an unphysiologically high fatty acid-to-albumin molar ratio of 6 (see above), palmitate had no significant

![FIGURE 8](image-url)

**FIGURE 8.** A: unbound fatty acid as a function of molar ratio to albumin. The physiological range is considered to be 1:1 to 2:1 (442). [Data from Cunningham et al. (105).] B: facilitation of GSIS from wild-type and \(Gpr40^{−/−}\) mouse islets by 5:1 oleate/albumin. Note the dramatic potentiation of second phase release. [Data from Ferdaoussi et al. (152).]
effect on basal cell respiration in the absence of glucose, but progressively enhanced high-glucose respiration and insulin secretion in parallel. Since β-oxidation is higher in low glucose than in high glucose, and substrate delivery exerts a high control over respiration in these cells, this is somewhat counterintuitive. Palmitate blunted the glucose-induced elevation in NADH, which reflects the matrix rather than cytosolic pool, and had no significant effect on the basal, or glucose-dependent increase in, whole cell ATP/ADP ratio. This study therefore does not resolve the paradox that a seemingly robust β-oxidation in low glucose does not have bioenergetic consequences similar to those seen with exogenous glucose, pyruvate, or other secretagogues.

The key bioenergetic experiments would be to quantify the acute effect of physiologically relevant fatty acid concentrations on the components of Δp and ΔGp in low glucose, while rigidly excluding the possibility of nonphysiological “uncoupling” effects. While the deleterious effects of chronic fatty acid exposure (“lipotoxicity”) have been detailed (e.g., Refs. 269, 277), I have been unable to find an article in which the acute effects of a fatty acid-to-albumin molar ratio not exceeding 3:1 have been compared with high glucose in terms of respiration, Δψnr and ΔGp.

VIII. THE COUPLING FACTOR CONCEPT

The origin of the concept of metabolic coupling factors (reviewed in Refs. 315, 371, 420) was discussed previously. Essentially, a coupling factor may be defined as an intracellular metabolite that appears to enhance in vitro first (triggering) or, predominantly, second (amplification) phase insulin secretion by a mechanism other than bioenergetic control of the KATP channel. Many putative coupling factors are related to mitochondrial metabolism; in a recent review, Prentki et al. (420) listed no fewer than 18 candidates, emphasizing that this was an unsatisfactory and confusing state of affairs, exacerbated by the general lack of molecular targets (420). The authors further distinguished between regulatory metabolic coupling factors that may control metabolic pathways associated with GSIS, and effectory metabolic coupling factors proposed to act on ion channels or the exocytotic mechanism itself in either phase (420). Their first category includes citrate, NAD(H), malonyl-CoA, GTP, long-chain (LC) acyl-CoAs, glutamate, and adenine nucleotides, while ATP, monoacylglycerol, and NADP(H) are among their effectory factors. I would exclude the adenine nucleotides from this list in an attempt to distinguish “metabolic” from “bioenergetic” control.

How does a metabolite become classified as a candidate coupling factor, rather than a constitutive component of the cell’s metabolism? First, it appears necessary that its concentration should be insufficient, after a conventional period of low glucose starvation, for the optimal support of GSIS, but is replenished in response to elevated glucose with a time course that matches, or precedes the second phase of insulin secretion. In other words, the level of a candidate coupling factor and the rate of insulin secretion would increase in parallel, following a low to high glucose step. Of course, even when this is found it does not indicate causality, and is not a sufficient criterion, since almost without exception, β-cell glycolytic and mitochondrial metabolites increase in concentration following a low to high glucose transition (FIGURE 7B). A second criterion is that at least some of the change in concentration of the candidate should occur in the cytosolic compartment. Third, it should be possible to demonstrate a facilitation of secretion paralleling an increase in the cytosolic concentration of the factor with no accompanying change in ΔGp, to control for a primary bioenergetic explanation for the facilitation. Fourth, a decrease in concentration of the factor by knockdown or inhibitors, again with no effect on ΔGp, should cause a parallel decrease in secretion.

Two extremes can be envisaged for a candidate coupling factor: at one, the concentration of the factor would be critical but its turnover relatively slow, similar to a classical signaling molecule such as cAMP. At the other extreme, the factor might be stoichiometrically utilized so that the rate of supply would be of prime importance, as with a conventional metabolic pathway. Metabolic control analysis would be extremely helpful in this context, since, in the absence of such information, it is not valid to dismiss a candidate pathway because it is slow [e.g., the pentose phosphate pathway for the generation of cytosolic NADPH (310)], or because partial knockdown of a constituent enzyme is without effect on insulin secretion, particularly if knockdown is based on mRNA levels rather than protein or activity.

As will be reviewed later, a key feature of GSIS, both in vivo and from the isolated islet, is its pulsatile nature, with a typical periodicity of 2–6 min. Investigation of associated oscillatory metabolisms has generally not been a focus of research into coupling factor pathways, particularly since synchrony is lost in the dissociated primary cell and insulinoma population preparations commonly employed for these investigations. For that reason, as far as possible, we shall consider “coupling factor” and “oscillatory” mechanisms separately.

A. Citric Acid Cycle Derived Coupling Factors, Pyruvate Carboxylase, and Anaplerosis

The transition from low to high glucose initiates a dramatic increase in the intracellular concentrations of multiple glycolytic and mitochondrial metabolites. Regardless of the assay technique, it is important to use extraction techniques that are sufficiently rapid to preclude artificial changes. This is particularly essential for highly labile bioenergetic
intermediates, such as the adenine nucleotide and nicotinamide nucleotide pools, which can change their equilibria within seconds. Conventional centrifugation produces an anoxic pellet, and if, as in one recent example, cells equilibrated with 2.5 or 12 mM glucose are each reported to contain equimolar ATP and AMP (187), one can confidently reject the results as artifactual.

With this proviso, metabolomic studies reveal 5- to 20-fold increases in metabolites such as citrate, 2-oxoglutarate, and malate during the period following a low to high glucose transition (FIGURE 7B), although absolute quantitation is rarely applied (188, 230, 490). It should be noted that while the low to high glucose transition has attracted a vast range of papers, the reverse process, the depletion of metabolites on the return to basal glucose, has been largely neglected. An increase in citric acid cycle intermediates in response to increased metabolism is not unique to β-cells. Vigorous exercise can cause a ninefold increase in the content of citric acid cycle intermediates in human skeletal muscle (466).

Mitochondria are generally depleted in citric acid cycle intermediates on isolation, which is why malate is commonly added together with pyruvate, glutamate, etc. Interestingly, pyruvate-supported respiration of isolated rat skeletal muscle mitochondria is enhanced, in the absence of added malate, by HCO$_3^-$ in the presence of ATP, correlating with anaplerotic refilling of the citric acid cycle via pyruvate carboxylase (110). One should therefore be open to the possibility that the anaplerosis observed in islets and β-cells in high glucose is simply the restoration of “normal” levels of the intermediates following in vitro starvation during a low glucose preincubation (FIGURE 7C). Does anaplerosis simply restore bioenergetic function by replenishing levels of citric acid cycle intermediates depleted by hypoglycemic preincubation, and, most importantly, how significant is it in humans where fasting glucose does not normally fall below 4 mM?

Fransson et al. (157) observed that phenylacetate (PAA), a supposedly selective PC inhibitor, depressed both insulin secretion and the glucose-induced increase in rat islet ATP/ADP ratios, while having no effect on exocytosis evoked by square-wave depolarization, and concluded that there was a bioenergetic component to the dependency of insulin secretion on PC-mediated anaplerosis. It should, however, be noted that PAA is incompletely characterized as a specific PC inhibitor. The observation that a 50% decrease in PC expression in INS-1 832/13 cells had little effect on metabolism or GSIS (239) cannot be interpreted without a metabolic control analysis to quantify flux control coefficients for this pathway.

What is the fate of the “excess” oxaloacetate generated by PC? The statement can be found in the literature that the citric acid cycle cannot act as a store of intermediates, and that cataplerosis (the subsequent removal of excess intermediates for the biosynthetic processes) must balance anaplerosis (474). However, following the transition from low to high glucose in the β-cell, the substantial increase in the levels of intracellular citric acid cycle intermediates refutes this. The most dramatic increases are in citrate, isocitrate, and malate which showed a time-dependent increase (491) reaching 5- and 20-fold within 60 min in both rat islets and INS-1 832/13 cells, while levels of succinate were largely unchanged (FIGURE 7B) (489). Incidentally, these non-steady-state conditions mean that classical metabolic control analysis would be difficult to perform under high-glucose conditions before a new steady state is achieved, i.e., after ~1 h.

In the absence of rapid subcellular fractionation, it is not currently possible to determine the proportion of these intermediates retained by the mitochondria. The presumption in the literature is that the majority are exported to the cytosol to facilitate the formation of coupling factors. However, there is a tendency to overlook the role of the pH gradient across the mitochondrial membrane, which increases in high glucose (536), and tends to retain malate and, particularly, citrate within the matrix (444). With the estimate by Wiederkehr et al. (536) that the trans-mitochondrial ΔpH in rat islet β-cells increases from 0.13 to 0.51 pH units from low to high glucose, the equilibrium matrix-to-cytosol citrate gradient will increase from 2.5:1 to 33:1 (proportional to the third power of the proton gradient), while that for malate increases from 1.8- to 10-fold (second power relationship). Actual results determined for heart and liver were ~10:1 and 4:1 for tri- and dicarboxylic acids, respectively (484, 540). For there to be net flux to the cytosol the gradients must be even greater. This suggests that a significant proportion of the additional citrate, and perhaps malate, generated by anaplerosis will be retained within the matrix, rather than exported. The technique of $^{13}$C-NMR mass isotopomer analysis has proven invaluable for the quantification of anaplerotic pathways in the β-cell (92, 240, 303). Briefly, cells are equilibrated with uniformly labeled [$^{13}$C]glucose; the labeled pyruvate enters the citric acid cycle through either PDH or PC, and analysis of the pattern of $^{13}$C labeling in the carbons of glutamate (equilibrating with 2-oxoglutarate via glutamate dehydrogenase) allows an estimate to be made of the relative flux through the two entry points. For INS-1 832/13 cells in low glucose, anaplerotic flux via PC and glutamate dehydrogenase accounted for 40% of the total citric acid cycle flux (92).

Comparison of high secreting with low secreting INS-1 cell lines (490), enhancing anaplerosis by addition of cell-permeant methyl malate (210), or inhibiting PC with PAA (157) in each case revealed a strong correlation between GSIS and the extent of anaplerosis via PC. While correlation
does not prove causality, these and related results support models in which anaplerosis produces conditions, or intermediates, conducive to exocytosis, although in each case a coupling factor hypothesis should exclude effects on the basic mitochondrial bioenergetic processes controlling $\Delta G_p$.

### B. Metabolic Cycles

As an outside observer of the $\beta$-cell field, I lack the competence to adjudicate in controversies as to the role of metabolic cycles in GSIS that are based on the selection of mouse strains or cell lines, the extent of knockdown or overexpression or the choice of experimental conditions. However, the extensive and frequently contradictory literature sometimes overlooks some fundamental bioenergetic principles and observations that are within the remit of this review and provide the focus of the following sections. The major metabolic cycles proposed to lead to the generation of coupling factors (FIGURE 7A) are discussed below.

1. **Malate/pyruvate cycles**

The 20-fold increase in intracellular malate in both rat islets and INS-1 832/13 cells following elevated glucose (489) is consistent with the anaplerotic generation of oxaloacetate and its equilibration with the matrix NAD/NADH pool via mitochondrial malate dehydrogenase. Malate can be converted to pyruvate by malic enzymes, of which $\beta$-cells can possess three isoforms: ME1 (cytosolic, NADP-linked), ME2 (mitochondrial, NAD or NADP-linked), and ME3 (mitochondrial, NADP-linked) (208, 311, 449). A pyruvate/malate cycle involving ME1 could generate cytosolic NADPH (FIGURE 7A). Cycles involving matrix-located ME2 or ME3 would act merely as an ATP-dissipating futile cycle (if NAD-linked), or an ATP-dependent matrix transhydrogenase (if NADP-linked). It is difficult to understand how this last would be important, given that most hydrogenase (if NADP-linked). It is difficult to understand how this last would be important, given that most hydrogenase (if NADP-linked).

2. **Pyruvate/citrate cycle**

The “pyruvate/citrate” cycle (150) (FIGURE 7A) involves the “forward” metabolism of oxaloacetate and the net generation of citrate in excess of that required for the steady-state operation of the citric acid cycle. Citrate would be exported to the cytosol (against pH gradient, as discussed above) by

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Each turn of the cycle would require the input of two pyruvates (via PDH and PC, respectively), the regeneration of one pyruvate and the net formation of one acetyl CoA. $^{13}$C-NMR studies on INS-1 832/13 cells indicated that pyruvate cycling in low glucose amounted to $\sim 30\%$ of the flux through the citric acid cycle, and that this increased to $>100\%$ in 12 mM glucose, averaged over a 4-h incubation (303; see also Ref. 92). I have a conceptual difficulty with these results, perhaps due to my unfamiliarity with the methodology. An anaplerotic/catablerotic pathway by definition produces a product, in the case of the pyruvate/citrate cycle, cytosolic NADPH, and AcCoA. In lipogenic tissues, these are precursors for fatty acid synthesis: AcCoA carboxylase generating malonyl CoA, while NADPH is required at two reductive steps. De novo fatty acid synthesis would seem to be the only outlet for the products (NADPH and malonyl-CoA) of a pyruvate/citrate cycle. However, fatty acid synthase is poorly expressed in rat islets (72), while data from INS-1 832/13 cells suggested that only $\sim 2\%$ of the total glucose utilized in high glucose was incorporated into lipid (248), although Macdonald and coworkers (313, 317) presented qualitative evidence that islets and INS-1 cells perform significant fatty acid synthesis. Note that even if a pyruvate/citrate cycle were to generate stoichiometric lipids, this would not in itself clarify a mechanism for the cycle to facilitate GSIS.

3. **Pyruvate/isocitrate cycle**

In this proposal (FIGURE 7A), citrate would again be exported from the matrix but would be converted to isocitrate via cytosolic aconitase (248). The cytosolic isocitrate pool would also be augmented by the direct transport of isocitrate, which is also transported on the tricarboxylic acid carrier. Cytosolic NADP-linked isocitrate dehydrogenase (IDH1) would then generate 2-oxoglutarate together with NADPH. In contrast to the pyruvate/citrate cycle, this would result in the net generation of reduced pyridine nucleotide. Finally, the 2-oxoglutarate would rejoin the matrix citric acid cycle via the 2-oxoglutarate carrier. Since this loop parallels, and reenters, the matrix citric acid cycle, it is free of the logistic problems with the pyruvate/citrate cycle.
discussed above, and could account for the massive increase in citric acid cycle intermediates when glucose is raised. Cytosolic isocitrate dehydrogenase is required for the cycle, but while Jensen et al. (240) reported that its suppression partially inhibited GSIS in INS-1 832/13 cells and rat islets, Guay et al. (195) came to an opposite conclusion, namely, that IDH1 is a negative regulator of GSIS.

4. Glutamate as a candidate coupling factor

In 1999, Maechler and Wollheim (326) proposed that cytosolic glutamate could function as a mitochondrially derived coupling factor to enhance GSIS. Addition of 5 mM cell-permeant dimethylglutamate caused a leftward shift in the glucose dependency of GSIS but, in contrast to mono- methyl succinate, did not hyperpolarize ΔΨm (326). This does not, in itself, demonstrate that physiological levels of cytosolic glutamate act as coupling factors. With the assumption that the ester has no effect per se (as has been suggested for methyl pyruvate, Ref. 136), the finding rather indicates that an in vitro increase in cytosolic glutamate facilitates a step between ΔΨm and release. The authors utilized Staphylococcus α-toxin to permeabilize INS-1 cells. In the presence of 0.5 μM Ca2+ and ATP, succinate and glutamate each potentiated insulin secretion. The dicarboxylates also appeared to elevate intracellular ATP, although the relevance of this for a permeabilized preparation in an ATP-containing medium is questionable. The V-ATPase inhibitor bafilomycin reversed the effect of glutamate, and the conclusion was that glutamate uptake into secretory granules was in some way facilitating exocytosis.

The Maechler paper immediately attracted controversy. While the authors found a fivefold increase in glutamate within 30 min in INS-1E cells and human islets, other workers (49, 314) found no correlation between islet glutamate levels and insulin secretion with a variety of secretagogues, while metabolite profiling of INS-1 832/13 cells showed no significant change in whole cell glutamate levels between low and high glucose (332). The reason for these discrepancies is unclear. Carobbio et al. (77) reported that conditional β-cell specific deletion of glutamate dehydrogenase reduced insulin secretion; however, this would have been predicted from the widespread metabolic and bioenergetic consequences.

Some of the apparent controversy may be semantic; the general understanding of a coupling factor is of a compound whose concentration under low glucose conditions is insufficient to facilitate secretion, but rises in response to elevated glucose sufficiently to augment GSIS, the ATP/ADP ratio being the classical example. If, however, the levels of cytosolic glutamate are sufficient to allow uptake into granules even under low glucose conditions, then the amino acid might be rather considered as a constitutive, “permissive,” component of the exocytotic process. Thus a recent study has indicated that insulin secretory granules express a vesicular glutamate transporter VGLUT3 (241), and more controversially, a “miss-targeted” plasma membrane glutamate transporter EAAT2 (166), while Gheni et al. (176) presented evidence that incretin-induced enhancement of GSIS required glutamate uptake into secretory granules. Designation of glutamate as a coupling factor, as defined earlier, may therefore not be appropriate.

C. Fatty Acid-Derived Coupling Factors

From a simple bioenergetic standpoint, the acute ability of exogenous fatty acids to stimulate insulin secretion is paradoxical (85, 189, 368, 421). In low glucose, when significant β-oxidation can occur (see above), added fatty acids are essentially ineffective, whereas in the presence of high (~17 mM) glucose, when β-oxidation is severely restricted by malonyl-CoA inhibition of CPT-1, the addition of palmitate and stearate, for example, at a physiologically relevant molar ratio to albumin of 3:1, can virtually double the 90 min insulin release from rat or human islets evoked by 16.7 mM glucose (189).

This has led to a focus of research into alternative, nonoxidative, roles of fatty acids and the long-chain acyl-CoA (LC-CoA) species that accumulate upstream of the CPT-1 block, as facilitators of insulin secretion by pathways other than ATP generation (reviewed in Refs. 370, 420). Three fatty acid-mediated coupling pathways have been proposed to facilitate GSIS, involving, respectively, a plasma membrane G protein fatty acid receptor, GPR40/FFA1, a glycerolipid/free fatty acid cycle, and effects mediated by long-chain acyl-CoA (390). The intricacies of these pathways, which are not mutually exclusive, have been extensively reviewed and critiqued (202, 322, 420) and are beyond the remit of this review, but raise relevant questions concerning the conditions required to observe effects ascribed to coupling mechanisms, particularly since excessive concentrations of fatty acids and long-chain acyl CoA esters can introduce artefacts.

1. Malonyl CoA and carnitine palmitoyl transferase-1 control of β-oxidation

The outer mitochondrial membrane carnitine-palmitoyl transferase-1 (CPT-1) is responsible for converting LC-CoA into acylcarnitine for transport into the matrix via the acylcarnitine/carnitine carrier, and exerts high flux control over fatty acid catabolism. CPT-1 is inhibited by malonyl CoA, an intermediate in fatty acid synthesis, thus limiting futile cycling between fatty acid synthesis and β-oxidation. β-Cells express the liver (L) form of the enzyme, characterized by a lower sensitivity to malonyl CoA, half-maximal inhibition requiring 2 μM (456). β-Cell fatty acid oxidation is extensively inhibited in high glucose (332, 407, 456), correlating with an increase in malonyl CoA, and resulting in an accumulation of LC-CoA. As discussed in the context
of a pyruvate/citrate cycle, malonyl CoA is formed from cytosolic acetyl CoA via acetyl CoA carboxylase-1 (ACC1), and its steady-state level will be determined by the relative activities of the carboxylase, fatty acid synthesis, and malonyl CoA decarboxylase (97, 364).

The pyruvate/citrate cycle focuses on anaplerotic metabolism underlying enhanced malonyl CoA levels in high glucose; however, acetyl CoA carboxylase is a classic target for AMPK (468), accounting for the bioenergetic control of fatty acid synthesis in multiple tissues (206). A valid point, therefore, is to what extent malonyl CoA control of CPT-1 may be primarily controlled by the cell’s bioenergetic status, via AMPK, rather than by the cataplerotic supply of cytosolic AcCoA by a pyruvate/citrate cycle, particularly since there appears to be no continuous output from the cycle as commonly formulated (FIGURE 7A). Energetic control makes physiological sense, allowing fatty acids to contribute to the support of basal, nonsecretory, bioenergetic functions in low glucose, preventing ΔGp from collapsing to levels below those required for normal cellular maintenance, while minimizing their bioenergetic contribution under high glucose conditions when they could compromise the precision of glucose-mediated bioenergetic control.

2. GPR40/FFA1

A plasma membrane fatty acid receptor GPR40/FFA1 (209, 236, 333) is highly expressed on β-cells and is believed to act predominantly (but not entirely, Ref. 209) via Gαq/11 and phospholipase C to elevate [Ca2+]c (70) and potentiate insulin secretion. As with other “nutrient sensing” receptors, the implication is that its affinity for the agonist (primarily long-chain fatty acid) is such that active site occupation, and hence activity, can be modulated by physiological variations in plasma unbound fatty acid (143). Richieri and Kleinfeld (442) estimated population average unbound free fatty acid concentrations in human serum of 6.8 and 8.3 nM, respectively, in nonfasting and fasting subjects. The assay has been generally accepted (30) and is in reasonable agreement with independent determinations of fatty acid binding to albumin (FIGURE 8A) (105). Physiology thus places severe restraints on the design of meaningful in vitro investigations into the role of GPR40.

GPR40 knockout mice display little or no phenotype (282), except under conditions of massive glucose overload (a 19 mM hyperglycemic clamp) when second-phase insulin secretion was reduced by 50% (15), or in response to intravenous intralipid plus heparin, which generated a massive peak serum fatty acid to albumin molar ratio close to 10:1. In this grossly nonphysiological condition, the massive insulin secretion evoked by intralipid was halved in the knockout (282). In vitro, similar extreme conditions have been frequently employed. The original report by Itoh et al. (236) reported EC50 values for palmitate of ~5 μM in the absence of albumin, while effects requiring 10 μM total fatty acid were greatly reduced in the presence of 1 mg/ml albumin. Schnell et al. (472) observed oleate-induced oscillations and a sustained increase in [Ca2+]c in primary mouse β-cells in the presence of 5 mM glucose; however, a molar ratio of 6:1 seemed to be required. El-Azzouny et al. (144) performed an exhaustive study of the bioenergetic and metabolic consequences of acute fatty acid exposure of INS-1 cells, including the use of a GPR40 antagonist, but again used a high 6:1 molar ratio. Ferdaoussi et al. (152) compared islets from wild-type and Gpr40−/− mice, concluding that GPR40 was responsible for ~50% of the oleate-mediated enhancement of second phase secretion (FIGURE 8B), but utilized a 5:1 molar ratio. It is possible that some of the uncertainty as to the mechanism mediated by the receptor results from the use of supraphysiological unbound fatty acid concentrations.

3. Long-chain acyl-CoA esters

The malonyl-CoA mediated inhibition of CPT-1 and resultant increase in long-chain acyl-CoA esters in high glucose led to the proposal that the latter may contribute to the fatty acid facilitation of GSIS as coupling factors (reviewed in Ref. 548). If CPT-1 inhibition is an essential step for the accumulation of long-chain acyl-CoA, it would be predicted that decreasing the level of malonyl-CoA by overexpressing malonyl-CoA decarboxylase would diminish the inhibition of β-oxidation and adversely affect GSIS. However, decarboxylase overexpression in INS-1 832/13 cells sufficient to lower malonyl-CoA accumulation and facilitate β-oxidation had no effect on glucose-stimulated insulin secretion (364).

A multiplicity of LC-CoA targets potentially capable of influencing secretion have been proposed from in vitro studies (reviewed in Refs. 97, 148, 548). These include the ANT (88), the tricarboxylic acid carrier, protein kinase C, Ca2+-ATPase, hormone-sensitive lipase, and the KATP channel (475), as well as several acylation targets and binding to nuclear transcriptional factors. The critical importance of employing physiologically relevant concentrations of amphipathic compounds extends to studies involving long-chain acyl-CoA esters (117, 118, 229). LC-CoA species partition extensively into membranes and affect function. Many cells contain fatty acid binding proteins (FABP) with KD values close to 1 μM, as well as specific acyl-CoA binding proteins (ACBP) with dissociation constants in the low nanomolar range, leading to the conclusion that the actual free LC-CoA concentration in the cytosol of these cells may normally be in the range 1–10 nM as long as the binding protein is in molar excess (148). The use, for example, of 20 μM oleoyl-CoA to modulate the KATP channel (475) clearly falls outside these constraints. As reviewed by Faergeman and Knudsen and co-workers (148, 265), “most of the reported effects of LC-CoA’s have been obtained by the addition of acyl-CoA directly, without a binding protein.” When binding is taken into account, many of these effects...
are considered by this group to be artifactual, with substrate provision to CPT-1 being one of the few actual physiological functions. To take one example, while Hu et al. (229) found that palmitoyl-CoA enhanced triglyceride lipase activity in islet lysates, the ester was inactive in the presence of equimolar ACBP, throwing doubt on the significance of the mechanism. The subcellular distribution of LC-CoA esters is also relevant. In heart, >90% of the total free and esterified CoA appears to be mitochondrial (148). The residual cytosolic CoA pool must be shared between free CoA, acetyl-CoA, malonyl-CoA, acetoacetyl-CoA, hydroxymethylglutaryl-CoA, succinyl-CoA, and LC-CoA species.

4. Glycerolipid/fatty acid cycling

The full complexity of the proposed glycerolipid/free fatty acid cycling in the control of insulin secretion has been recently reviewed (419). In essence, long-chain acyl CoA esters generated by acyl CoA synthase on the ER and outer mitochondrial membrane is diverted from β-oxidation by the malonyl-CoA inhibition of CPT-1 to be instead esterified to glycerol-3-phosphate to form sequentially lysophosphatidate, phosphatidate, and mono-, di-, and triglycerides, with concomitant hydrolysis to glycerol and fatty acid completing a dissipative cycle, utilizing ~7 ATP molecules. Each of these lipid intermediates, except triglycerides, has been considered as a candidate coupling factor (419), although the lack of clarity concerning targets and mechanisms is problematic.

D. Conclusion: Coupling Factors

There is general agreement, encapsulated by Prentki et al. (420), that the current understanding of metabolism-based signaling processes in the β-cell is unsatisfactory, in terms of both target and mechanism. The coupling factor concept appears to be unique to the β-cell field, and has evolved from the need to explain the biphasic kinetics of insulin secretion seen when cells or islets equilibrated under conditions mimicking borderline hypoglycemia, ~3 mM, are suddenly exposed to distinctly hyperglycemic conditions, typically 11–16 mM glucose. The problem is that a multitude of metabolic, ionic, and bioenergetic parameters, in addition to insulin secretion itself, readjust in response to this transition with a variety of response times. Unraveling this non-steady-state condition has proven formidably complex. If we accept the questionable physiological significance of the glucose step, then a definition of a metabolic coupling factor would appear to be a metabolite whose concentration has to increase following the transition to high glucose, to support optimal GSIS, and in particular the delayed, second phase, release. As I have tentatively suggested (FIGURE 7), an alternative interpretation is that the “starved” cells are metabolite depleted and that the subsequent exposure to high glucose results in the simple repletion of intermediate levels required for optimal metabolism.

Fatty acid-mediated coupling pathways fall into a different category. Their dramatic effects on second-phase insulin secretion (FIGURE 8) remain to be fully elucidated, but an important constraint is to work within physiological free concentration ranges, both in terms of extracellular fatty acid addition and the modeling of cytosolic free acyl-CoA levels.

IX. OSCILLATORY PARAMETERS

The human pancreas contains ~1,000,000 islets, each with ~1,000 β-cells containing some 10,000 secretory granules. In response to elevated glucose, insulin release detected in the portal vein oscillates with a period of 5–6 min (488). While no single mechanism appears to account for all aspects of the oscillatory entrainment of the entire population of islets in the pancreas in the absence of physical contact, a plausible mechanism involves a negative-feedback loop involving the liver, with some combination of insulin-mediated hepatic glucose uptake and/or glucose release during peaks and troughs, respectively, of insulin secretion (516, 550). The model is not without problems, notably in the ability of the perfused pancreas to maintain insulin secretory oscillations, suggesting the existence of a hypothetical intrapancreatic pacemaker (494). Additionally, Cunningham et al. (104) observed oscillatory secretion from groups of 50–100 isolated islets confined in a small column, consistent with an extracellular paracrine messenger. However, no such messenger has been identified, and others have found no synchrony between individual islets in a group superfused with constant glucose (124). The role of neurons projecting to the pancreas may also be significant (263). Pulsatile secretion appears to be central to the precise ability of insulin to restore basal glucose following a meal, preventing chronic hyperglycemia, or the even more dangerous acute hypoglycemia, and its disruption can be an early sign in the development of T2D (416).

The extreme sensitivity of insulin immunoassays allows release from single islets to be monitored with high temporal resolution. In the presence of constant elevated glucose, islets retain an autonomous oscillator controlling insulin secretion with a typical periodicity of 2–6 min (43, 107, 550). A recent representative study employed a microfluidic device to monitor insulin secretion from single isolated mouse islets following a 3–11 mM step increase in glucose (FIGURE 8A) (550). The study was interesting in that insulin secretion followed the classic time course of first- and second-phase release by varying the amplitude, rather than the frequency, of the individual pulses (FIGURE 2B) (see also Ref. 43).

If a single cell in the intact islet is patched, KATP channel inhibition by elevated glucose is seen to depolarize Δψp to a threshold at which bursts of action potentials are seen, at a typical frequency of 12 Hz, separated by quiescent periods.
The bursts repeat with a periodicity of \( \approx 5 \text{ min} \) (FIGURE 9C), and the proportion of burst relative to rest phase increases with glucose concentration, until bursting is continuous at very high glucose. A cycle of burst and rest is termed a “slow” oscillation.

Since insulin secretion is a collective property of the entire islet, pulsatile release suggests that electrical activity is synchronized throughout the islet. This can be detected noninvasively as extracellular field potentials with multi-electrode arrays (283, 410, 473), paralleling more conventional investigations with cell-attached patch electrophysiology. Synchronicity can additionally be studied with high temporal resolution imaging, allowing the detection of waves of depolarization. A new generation of photo-induced electron transfer voltage (VF) probes, in which electron transfer to a linked transmembrane quencher (and hence the extent of fluorescence quenching) is dependent on \( \Delta \Psi_p \), has opened the possibility of optical imaging of potential changes with a sub-millisecond time constant (353). Dolensek et al. (126) utilized this technique with an acute mouse pancreatic slice to detect depolarization bursts (but not high-frequency action potentials) with high temporal resolution simultaneously from multiple \( \beta \)-cells within an in situ islet. In response to 12 mM glucose, bursts with a median duration of 2.5 s spread from the periphery throughout an islet with a velocity of \( \approx 70 \mu m/s \), requiring a little over 1 s to traverse the islet.

Action potential firing triggers voltage-activated \( Ca^{2+} \) channels (VACCs) and elevates \([Ca^{2+}]_c\). Ravier et al. (437) observed a high degree of synchrony between slow \([Ca^{2+}]_c\) oscillations monitored simultaneously from multiple regions of a mouse islet, correlating with pulsatile insulin secretion. Notably, synchrony was disrupted in islets from \( ob/ob \) mice. High temporal resolution imaging revealed waves of elevated \([Ca^{2+}]_c\) propagate across the mouse islet at a mean velocity of \( 69 \mu m/s \) (41), identical to the velocity of depolarizing bursts reported by Dolensek et al. (126). While this would be expected if depolarization triggers \( Ca^{2+} \) entry, it does provide validation for the methodologies used in the two studies. Parallel monitoring of \([Ca^{2+}]_c\) from islet regions and \( \Delta \Psi_p \) from single cells revealed calcium oscillations synchronized to the depolarizing bursts (38, 180, 344). High-frequency bursts of action potentials result in a smooth elevation in \([Ca^{2+}]_c\) that returns to baseline with a \( t_{1/2} \) of \( \approx 1 \text{ min} \) on termination of the burst, consistent with removal of \( Ca^{2+} \) from the cytosol, by some combina-
tion of expulsion from the cell, uptake into ER, and sequestration by mitochondria (38, 180, 469). The pulsatility of insulin secretion can thus be accounted for by the periodic activation of VACCs. However, one puzzling feature, at least to the author, is an apparent disconnect between the periodicity of insulin secretion from single islets, which at roughly 5 min is reported to be rather insensitive to glucose concentration (43, 107, 550), and that of bursting, the frequency of which can be glucose-dependent and continuous in the presence of very high glucose (47, 160, 555). Burst termination has been ascribed to the activation of slow 
Ca$^{2+}$-activated 
Ca$^{2+}$ channels as [Ca$^{2+}$], accumulated during a burst (18, 131, 552).

A. Role of Gap Junctions

The detailed architecture of the islet and the extent and mechanism of cell-to-cell communication, “connectivity” (458), is key to an accurate understanding of the fine control of insulin secretion. This is an area in which techniques by which the responses of individual cells within the islet can be determined (i.e., based on electrophysiology and/or fluorescence imaging) can provide invaluable information, whereas this level of information is lost in those metabolic studies that report averaged parameters from the entire islet, particularly in view of the heterogeneity of cell type and their distinct architecture (225). Gap junctions are critical mediators of electrical communication, rather than diffusion of cytosolic messengers (553). The relatively limited gap junctional conductance (436, 481) and for buffering the inherent heterogeneity in responsiveness of individual cells (40). Whole cell patch-clamp studies with intact islets indicated that gap junctional conductance between cells is rather weak, although still sufficient for adjacent cells to contribute to the residual conductance when the $K_{ATP}$ channels in a patched cell are inhibited (553). It has been estimated that some six or seven adjacent $\beta$-cells contribute to the gap-junction-dependent conductance. Importantly, no transfer of a cytosolic marker, Lucifer yellow, was seen between cells, suggesting electrical communication, rather than diffusion of cytosolic messengers (553). The relatively limited gap junctional conductance would appear to be sufficient to entrain a group of cells sufficiently to prevent spontaneous firing in low glucose (40, 41, 553), while still allowing a graded, glucose-dependent response. Thus gap junction deletion in islets from Cx36$^{-/-}$ mice allowed some 50% of cells to show [Ca$^{2+}$], fluctuations in 5 mM glucose, whereas virtually all cells were silent in islets with intact gap junctions (40). Conversely, a synchronized wave could be initiated when a particularly glucose-sensitive cell exceeds the threshold and fires, sufficient depolarizing current being transmitted to the adjacent, subthreshold, cells for them in turn to fire. In this way, action potentials would be propagated throughout the islet by a form of chain reaction.

Gap junctional communication is clearly essential for the bundling of action potentials together to give prolonged bursts, as seen by the heterogeneous firing of individual islet $\beta$-cells in the absence of gap junctions (553), and the uncoordinated activity of dissociated primary (261) and insulinoma (175) cells. On occasion, a burst may break apart into discrete spiking (FIGURE 9), to produce so-called compound bursting (294, 406), while application of the nonselective K$^+$ channel inhibitor tetraethylammonium also disrupts bursting to produce discrete stochastic spiking.

Key questions include the mechanism(s) by which bursting and stochastic spiking are controlled, and these will now be discussed.

B. Bursting: Oscillations in Upstream Delivery, Downstream Dissipation, or Both?

An upstream, purely bioenergetic, model can be formulated based on self-sustaining oscillations in glycolytic and oxidative flux, produced by the accumulation of products that exert a negative feedback on earlier metabolic steps.

1. A glycolytic oscillator

The glycolytic pathway is capable of autonomous oscillation. Tornheim and Lowenstein (517) reported that a concentrated muscle cytoplasmic extract supplemented with ATP, NAD, and glucose underwent spontaneous metabolic oscillations with a period of ~10 min, involving large fluctuations in ATP, NADH, glycolytic intermediates, and pyruvate. Product activation of PFK-1 by fructose-1,6-bisphosphate (F1,6BP), leading to an enhanced $\Delta G_m$, decreased AMP, and consequent PFK-1 reinhibition, was considered to provide the basis for the oscillatory mechanism (517). The high sensitivity of the muscle (M-PFK-1) enzyme for F1,6BP was considered important for this mechanism. When the model was extended to the $\beta$-cell, INS-1 cells showed high concentrations of the M-form; however, problematically, rat islets appeared to possess only trace amounts of the M-isoform as judged by Western blotting (549).

A further layer of complexity is introduced by the bifunctional enzyme PFK-2/FBPase2 (phosphofructo-2-kinase/fructose-2,6-bisphosphatase), responsible for the synthesis and degradation of fructose-2,6-bisphosphate (F2,6BP) (21). F2,6BP overrides the negative feedback of PFK-1 by citrate and an elevated ATP/ADP ratio. Glucose increases the concentration of F2,6BP in islets, albeit rather modestly (21). Puzzlingly, AMP-kinase activation, which would be
most apparent in low glucose, also increases F2,6BP (21). F2,6BP acts to activate PFK-1 and to lower the threshold for glycolytic oscillations (292, 348). In addition to controlling F2,6BP levels, PFK/FBPase2 binds to, and activates, glucokinase, apparently by physical interaction rather than enzymatic activity (292).

An additional control locus in glycolysis is pyruvate kinase M2 (PKM2), whose oligomerization to an active form is facilitated by F1,6BP (28). Merrins et al. (350) constructed a FRET biosensor, “PKAR” to monitor the extent of oligomerization of PKM2, as a means of monitoring fluctuations in F1,6BP and hence glycolytic flux. Both MIN6 cells and mouse islets showed oscillations in emission from the construct, with periodicities of ~2 and 6 min, respectively. However, the peak-to-trough amplitudes of the oscillations were modest: in the case of the islet, 11% of that produced by a 10 to 2 mM glucose transition. Parallel monitoring of flavin reduction from nontransduced cells within the same islet indicated a linked redox potential oscillation in phase with the FRET indicator, suggesting that the electron transport chain responded to the implied glycolytic oscillations. The amplitude was 10–20% of that seen in a 10 to 0 mM glucose transition.

As discussed above, single islet insulin secretion monitored in a microfluidic device showed high-amplitude oscillations with a period of ~5 min in the presence of constant elevated glucose (550). Remarkably, the periodicity could be entrained by imposing small sinusoidal oscillations in applied glucose (550). Thus oscillations as little as ±0.75 mM on 11 mM glucose were able to entrain not only single islets, but also groups of islets (124). Peak [Ca^{2+}]_{i} followed peak glucose with a lag of 90 s and peak insulin secretion after 140 s. This suggests that quite modest variations in upstream metabolism can dramatically entrain insulin secretion, with intercellular synchrony being presumably still dependent on gap junctional communication. While mechanisms intrinsic to the islet were not excluded, the model provides persuasive support for an in vivo mechanism relying on negative feedback from liver glucose uptake (124).

These elegant studies indicate a glycolytic oscillator that is able to command all the downstream bioenergetic events leading to insulin secretion. The key transition from glycolysis to oxidative phosphorylation is at the mitochondrial pyruvate transporter and/or pyruvate dehydrogenase (“PT/PDH”), and a glycolytic oscillatory driver presumably implies that the concentration of cytosolic pyruvate delivered by glycolysis should oscillate in turn, sufficiently to control ΔGp. In MCA terms, PT/PDH should demonstrate significant elasticity to cytosolic pyruvate. The low to high glucose transition, or the addition of as little as 1 mM exogenous pyruvate, roughly doubles respiration [FIGURE 4A]. However, the average concentration of pyruvate in both rat islets and INS-1 832/13 cells increases more than 10-fold (489) [FIGURE 7]. MCA theory tells us that the elasticity of PT/PDH to cytosolic pyruvate will be dramatically decreased under these conditions, and, together with the buffering of cytosolic pyruvate by the greatly enlarged pool, makes it difficult to visualize how small fluctuations in glycolysis in high glucose could induce oscillations in cytosolic pyruvate of sufficient magnitude to control mitochondrial respiration without additional processes being involved.

2. Respiration

Mean islet mitochondrial respiration can be monitored by determining the extent of oxygen depletion in the core of an individual islet using an inserted oxygen microelectrode (250, 259, 395). Oscillations of ~10% can be detected in the oxygen tension recorded within individual mouse islets perfused with 10 mM glucose, suggestive of fluctuations of respiratory rate of a similar magnitude (249, 250, 395). Simultaneous monitoring of intra-islet glucose with a second implanted electrode was technically challenging, but showed both slow (~3 min) and fast (~20 s) oscillations in both parameters (250). Surprisingly, these were out of phase, i.e., when glucose depletion (and hence utilization) was maximal, oxygen depletion (and electron transport) was at a minimum. This could only occur if one or more glycolytic intermediates accumulated during the high glycolysis/low respiration phase and were subsequently oxidized. MCA would clearly be of value here. Simultaneous measurements of pulsatile insulin secretion from the single islet revealed a phase shift, such that peak respiration occurred during the rising phase of an insulin secretory oscillation.

While this and related studies indicate a linkage between respiration and secretion, they do not distinguish between an enhanced upstream delivery and an increased downstream demand. Remember that a cell respirometer experiment reports increased respiration in response to both upstream (glucose) and downstream (protonophore addition) perturbations [FIGURE 4]. However, cyclic respiratory acceleration was eliminated by K_{ATP} activation with diazoxide, suggesting that metabolic oscillations were not autonomous, but rather dependent on plasma membrane ionic events (259). This might be expected, since it is difficult to conceive of a mechanism that could maintain metabolic synchrony between hundreds of individual cells in the absence of a feedback from processes associated with bursting.

3. Δψ_{m}

It must always be born in mind that it is not valid to equate Δψ_{m} with the protonmotive force and that the ΔpH component of Δp is not only significant, but changes in parallel with Δψ_{m} in high glucose (536). I am not aware of any
studies in which \( \Delta p \) oscillations have been detected or related to other oscillatory parameters. To achieve the time resolution required to detect \( \Delta \psi_m \) fluctuations, probes such as TMRM or R123 are employed in quench mode, although these are difficult to quantify (380). Luciani et al. (305) observed small R123 fluorescence fluctuations of \( \sim 2\% \) in mouse islets, equal to \( \sim 12\% \) of the hyperpolarization seen in a 3–10 mM glucose step. An extensive investigation of \( \Delta \psi_m \) oscillations in the intact islet has been performed by Katzman et al. (253). The authors loaded islets from the Israeli sand rat with R123 in quench mode and observed populations of cells oscillating with high regularity with a periodicity of \( \sim 5 \) min. The approach did not allow the magnitude of the oscillations to be estimated, but provided a highly detailed analysis of the synchronicity, regularity, and distribution of the oscillations.

4. NAD(P)H autofluorescence

As discussed above, NAD(P)H autofluorescence partially reflects mitochondrial NADH and provides a rough qualitative assessment of changes in \( \Delta p \). Small \(( \sim 2\% \) oscillations in NAD(P)H signal with a period of 5 min were seen in single mouse islets in the presence of 10 mM glucose (305). To put the NAD(P)H oscillations in context, their peak-to-trough amplitude amounted to \( \sim 12\% \) of the sustained increase in autofluorescence in the same experiment during the transition from 3 to 10 mM glucose (305). NAD(P)H oscillations were broadly in phase with, and dependent on, \([Ca^{2+}]_c\) oscillations, but the rising phase of the autofluorescence started some 2 min prior to the initiation of the \([Ca^{2+}]_c\) pulse (or when \([Ca^{2+}]_c\) from the previous \(Ca^{2+}\) oscillation was almost restored to basal). An important semantic point concerning the interpretation of quasi-sinusoidal oscillations is whether the peaks represent energetic enhancements relative to the basal, nonoscillatory state, or whether the troughs indicate energetic depletions relative to basal. For example, in the above study, does the rising phase of the NAD(P)H fluorescence represent the initiation of a bioenergetic process driving the \([Ca^{2+}]_c\) oscillation, or merely a recovery to baseline after the energetic demand of a previous \(Ca^{2+}\) sequestration is completed?

5. Oscillations in the cytosolic adenine nucleotide pool

The most economical explanation linking upstream bioenergetic fluctuations to bursting would involve an increase in \( \Delta G_p \) sufficient to depolarize \( \Delta \psi_m \) beyond the threshold for action potential firing, followed by a subsequent lowering due to a feedback restriction on metabolism, and/or increased ATP demand associated with ion channel activation, \(Ca^{2+}\) handling, and secretion. If so, it should be possible to detect underlying oscillations in a \( \Delta \psi_m \)-related parameter. At this stage we shall only consider studies in intact islets. As discussed above, cytosolic ATP levels change only slightly in response to elevated glucose. Thus the FRET probe GO-ATeam1 (366) responded to an increase in glucose from 2.8 to 25 mM glucose with a 10–15% increase in relative fluorescence in mouse islets (FIGURE 5C) (506). The authors employed cross-correlation analysis but were unable to detect any periodic fluctuation in [ATP]c associated with \([Ca^{2+}]_c\) oscillations determined in parallel. In contrast, Li et al. (293) exploited TIRF to detect synchronized subplasma membrane Perceval oscillations with a period of \( \sim 5\) min in mouse islets in elevated glucose. Cross-correlation analysis with simultaneously imaged \([Ca^{2+}]_c\) showed that the cytosolic \(Ca^{2+}\) peaks were followed 30 s later by Perceval minima, consistent with a negative feedback on \(\Delta \psi_m\) from the bioenergetic demands of \(Ca^{2+}\) sequestration. It should be noted in passing that while the authors consider Perceval to monitor [ATP] rather than ATP/ADP, this may be due to a misinterpretation of effect of an adenylyl kinase inhibitor, as discussed previously.

The downstream consequences, but not the causes, of bursting can be investigated by imposing artificial burstlike depolarizing patterns on dissociated cells (510). Tarasov and Rutter (510) reported a drop in cytosolic ATP/ADP ratio monitored with Perceval when bursting was simulated by imposing repetitive square-wave depolarizations in perforated patch mode. The decrease in \(\Delta \psi_m\) was reversed on termination of the “burst” and replaced with a signal that rose above the prestimulation baseline for some 5 min (FIGURE 10A).

6. A role for mitochondrial \(Ca^{2+}\)?

While some early models focused on ER-mediated \(Ca^{2+}\) fluxes to explain bursting (48, 84), subsequent proposals have included mitochondrial \(Ca^{2+}\) handling. Matrix \(Ca^{2+}\)
accumulation in response to transiently elevated \([\text{Ca}^{2+}]_{\text{m}}\), can produce three sequential bioenergetic effects, firstly a fall in \(\Delta p\) during net uptake as a consequence of the utilization of the proton circuit to drive the accumulation, augmented by the utilization of cytosolic ATP to pump \(\text{Ca}^{2+}\) from the cytosol into the ER or out of the cell. This would be followed by a mitochondrial hyperpolarization due to activation of key dehydrogenases, and finally a return to baseline when \([\text{Ca}^{2+}]_{\text{m}}\) falls and the matrix unloads the excess \(\text{Ca}^{2+}\). In interpreting and critiquing these \(\text{Ca}^{2+}\)-dependent responses, it is essential to distinguish between changes in \(\Delta \psi_{\rho}\) and \(\Delta p\), particularly as the \(\Delta \rho\)H component of the protonmotive force is highly dependent on the relative uptake of \(\text{Ca}^{2+}\) and \(\rho\) (376).

The ability of mitochondria to show two sequential, opposing bioenergetic responses to \(\text{Ca}^{2+}\) uptake: partial depolarization during net accumulation, followed by hyperpolarization due to citric acid cycle activation, has been incorporated into proposals integrating mitochondrial \(\text{Ca}^{2+}\) transport with slow metabolic oscillatory hypotheses (9, 47, 121, 257, 258, 293, 327, 428). The finite kinetics of the mitochondrial uniporter and efflux pathways means that \([\text{Ca}^{2+}]_{\text{m}}\) begins to increase some 10–15 s after the elevation of \([\text{Ca}^{2+}]_{\text{c}}\) (179), ramps up during a burst or rapid trains of bursts, and subsequently decays to basal (510). Imposed bursts (e.g., 10 bursts of 25 “action potentials” at 5 Hz over 2.5 min) produced a distinct decrease in \(\Delta G_{\rho}\) monitored with Perceval (510), consistent with a drop in \(\Delta \rho\) during the net \(\text{Ca}^{2+}\) accumulation. A subsequent recovery and overshoot in Perceval signal following termination of the train of pulses (FIGURE 10) was consistent with \([\text{Ca}^{2+}]_{\text{m}}\)-mediated metabolic enhancement. Unfortunately, the experiment was terminated while the Perceval signal was still rising so that the final predicted component, the restoration of the prestimulus \(\Delta G_{\rho}\) to accompany release of the “excess” \(\text{Ca}^{2+}\) from the matrix, was not observed. Drews et al. (132) have raised the objection that \([\text{Ca}^{2+}]_{\text{m}}\)-mediated metabolic enhancement would provide a positive feedback incompatible with oscillations.

If matrix pyruvate availability exerts significant flux control, an additional feed-forward could result from the enhanced \(\Delta \rho\)H accompanying \(\text{Ca}^{2+}\) uptake into the matrix. Since pyruvate uptake into the matrix is an electroneutral cotransport of pyruvate– plus \(\rho\)H+, uptake might be facilitated by the \(\rho\)H gradient. In conclusion, while a simple mitochondrial \(\text{Ca}^{2+}\)-mediated cycle is attractive, phase relationships create a problem. A single \(\Delta p\) depolarization-hyperpolarization-return cycle can be envisaged in response to an imposed burst (510), but lacks a mechanism for the initiation of a burst after a quiescent interval and its subsequent termination. Some (259, 305) but not all (349) studies reported that clamping \(\Delta \psi_{\rho}\) hyperpolarized with diazoxide terminated metabolic oscillations, suggesting that \([\text{Ca}^{2+}]_{\text{c}}\) oscillations could feed back onto glycolysis via fluctuating ATP demand.

7. Is glycolysis needed for slow oscillations?

The synthetic substrate monomethyl succinate (MMS) is sufficiently hydrophobic to cross the \(\beta\)-cell plasma membrane and be subsequently hydrolyzed in the cytosol by nonspecific esterases to yield succinate (together with methanol). MMS is a secretagogue for islets from the rat, but not the mouse (213, 309, 311, 316), a distinction proposed to result from the absence of the anaplerotic malic enzyme from the latter (213). MMS induced \(\Delta \psi_{\rho}\) and \([\text{Ca}^{2+}]_{\text{c}}\) oscillations in the rat islets indistinguishable from those produced by glucose, as well as a “classic” first- and second-phase insulin secretion, and the authors concluded that “systems other than glycolysis might control these oscillations” (213). The study was extended to methyl pyruvate and \(\alpha\)-ketoisocaproate (KIC) (212). Both substrates supported regular “slow” \([\text{Ca}^{2+}]_{\text{c}}\) oscillations in single dissociated mouse islet cells. It should be noted, however, that Dahlgren et al. (107) failed to observe slow oscillations in intact mouse islets with these substrates.

C. Stochastic Spiking by Dissociated Cells: The Ionic Oscillator

Intercellular synchrony and the characteristic slow \(\Delta \psi_{\rho}\) bursting observed in intact islets are lost once islets are dissociated into single cells, and are not seen in insulinoma cell culture, whether due simply to the lack of gap-junctional contacts, or to the dilution of proposed diffusible factors. Instead, dissociated primary \(\beta\)-cells (211, 247, 487, 513) and insulinoma cell lines (175, 183, 221) display irregular heterogeneous \(\Delta \psi_{\rho}\) and \([\text{Ca}^{2+}]_{\text{c}}\) “spiking” in response to elevated glucose. It is notable that the pattern of \(\Delta \psi_{\rho}\) and \([\text{Ca}^{2+}]_{\text{c}}\) spiking seen, for example, in single INS-1 832/13 cells in the presence of glucose or other secretagogue (175) closely resembles that recorded from intact islets when bursting is disrupted by \(K^+\) channel inhibition with tetraethylammonium (FIGURE 9C) (469). This indicates that conductance through non-\(K_{\text{ATP}}\) potassium channels plays a critical role in the “bunching” of action potentials into discrete bursts in the intact islet.

Dissociated \(\beta\)-cells show considerable cell-to-cell variability in their responsiveness to glucose (414, 522) and are generally rather poor oscillators compared with cells in situ within the intact islet (417). It is a concern that this heterogeneity is frequently not quantified in the literature, and that, when selecting “representative” cells, those exhibiting robust regular oscillations tend to be chosen. Since the secretory pathways associated with oscillating and nonoscillating cells differ, not least in their \(\text{Ca}^{2+}\) channel use (175), major uncertainties can be introduced when correlating single-cell and population parameters. Automated calculation
of the proportion of oscillatory cells within a field, the amplitude of the \( \Delta \psi_p \) and \([Ca^{2+}]_c\) oscillations and the mean interspike interval among the cells in a field of view can help to avoid this (175).

1. The ionic oscillator is not driven by upstream bioenergetic fluctuations

At least in the case of INS-1 832/13 cells, stochastic oscillations in \( \Delta \psi_p \) and \([Ca^{2+}]_c\) can be initiated by pyruvate as effectively as by glucose (175, 183), eliminating any role of a glycolytic oscillator.

Small irregular glucose-dependent fluctuations in R123 fluorescence in single dissociated \( \beta \)-cells have been reported by Nunemaker and Satin (392), Kindmark et al. (262), and Krippevity-Drews et al. (272). However, Goehring et al. (183) increased the precision of the technique by focusing on the cytosolic TMRM signal in INS-1 832/13 cells metabolizing pyruvate, and searched for correlations between \( \Delta \psi_m \) and individual \([Ca^{2+}]_c\) spikes. In this study \([Ca^{2+}]_c\) spikes were followed by very small mitochondrial hyperpolarizations (\(<1 \text{ mV}\)), which would be inconsistent with a causal role of mitochondrial bioenergetics as a driver. In the same preparation, NAD(P)H autofluorescence increased transiently, peaking some 7% over baseline at 10 s following individual \( \Delta \psi_p \) spikes (183), consistent with a downstream response.

The detection of single cell ATP oscillations, regardless of their linkage to plasma membrane events, is not trivial. As discussed previously, cytosolic ATP concentration is an insensitive monitor of \( \Delta G_p \) (FIGURE 5A). Despite this, Kennedy et al. (258), using luciferase targeted to the subplasma membrane, reported that they “occasionally observed oscillations in [ATP]_pm, although such oscillations were rare.” In a subsequent paper, Ainscow and Rutter (9) were able to detect small oscillations in luciferase signal in primary luciferase-infected \( \beta \)-cells, although with a low signal-to-noise ratio. Human cells oscillated in both low and high glucose, while mouse cells oscillated in elevated glucose. Simultaneous analysis of \([Ca^{2+}]_l\) was not feasible, although the authors speculated that ATP elevation was the primary event enhancing \( K_{ATP} \) closure and \( Ca^{2+} \) entry, with the oscillatory cycle completed by a decrease in ATP due to the energetic demand of \( Ca^{2+} \) sequestration (9).

In contrast, Goehring et al. (183) targeted the related ATP sensor ATeam 1.03 to the mitochondrial matrix of INS-1 832/13 cells. Averaging 130 events showed that individual \([Ca^{2+}]_c\) spikes in the presence of pyruvate were accompanied by a transient 12% drop in matrix ATP, which subsequently recovered and overshot (FIGURE 10B). The limited time resolution did not allow the temporal relationship between the two parameters to be determined, but, on the assumption that matrix and cytosolic ATP concentrations changes in parallel, the direction of the change was consistent with a transient increased ATP demand resulting from a primary plasma membrane depolarization and \( Ca^{2+} \) entry, rather than a modulation of \( K_{ATP} \) activity, which would have required an increase in ATP.

2. The channels contributing to the ionic oscillator differ between preparations

The nature of the inward current responsible for depolarizing the cell to the threshold for action potential firing is unclear (452). The ion channels responsible for action potential firing are not conserved, but differ between insulinoma cells, rodent, and human \( \beta \)-cells (64, 452). L-type \( Ca^{2+} \) channels are primarily responsible for the depolarizing action potential stroke in mouse \( \beta \)-cells; the role of \( Na^+ \) channels, though present in these cells, was unclear (452). In human \( \beta \)-cells, \( Na^+ \) channels and \( L- \) and \( T- \) type \( Ca^{2+} \) channels contributed to action potential firing, although P/Q-type channels were most effective in mediating exocytosis, suggesting specific roles for channel subtypes (64).

The INS-1 832/13 cell is notable for the ion channels that are apparently not required. While the cells possess tetradotoxin-sensitive \( Na^+ \) channels, the toxin is virtually without effect on the oscillation parameters; this contrasts with MIN6 cells (389). Likewise, oscillations continue in the combined presence of 4-aminopyridine and tetraethylammonium (175) that between them inhibit >90% of the \( K_{ATP} \)-independent conductance of INS-1 cells (498). Surprisingly, \( \Delta \psi_p \) or \([Ca^{2+}]_c\) oscillations were not blocked by L-channel inhibitors, but T-channel inhibition suppressed both oscillations (175).

D. Model Building

The processes responsible for the initiation, duration, and termination of bursts as well as for the duration of the quiescent interburst interval are complex, contentious, and incompletely resolved. It may be necessary to distinguish to some extent between rodent and human islets, since bursting is less prevalent in the latter preparation (443), whether due to differences in ion channel population (64), islet organization (56), or practical difficulties in handling the human islets. The loss of slow bursting when rodent islets are dissociated (487) indicates that gap junctional contacts play a role in the grouping of action potentials into active and quiescent periods, and must be incorporated into proposed mechanisms.

Key observations that must be accounted for include the ability of the isolated islet to maintain synchronized oscillations in glycolysis and electron transport chain activity in the presence of continuous elevated glucose, the ability of small oscillations in glucose concentration to entrain the islet to different oscillation frequencies, and the role of gap junctions in the grouping of action potentials into bursts with intervening quiescent periods. A comprehensive and critical nonmathematical overview of the multiple experi-
mental and theoretical models that have been advanced to interpret the complex pattern of β-cell firing (e.g., Refs. 44, 45, 121, 159, 293, 391, 534) has recently been published (151). The most comprehensive current mathematical construction, the Dual Oscillator Model, proposes interactions between three modules: glycolysis, the mitochondrion, and the plasma membrane or \([\text{Ca}^{2+}]_{c}\) oscillation (439). In contrast, spiking, or fast bursting, would be governed by a separate plasma membrane electrical oscillator, generating \([\text{Ca}^{2+}]_{c}\) oscillations that feed into the mitochondria as discussed above.

It is, however, debatable to what extent a purely mathematical model, however ingenious, can fully encompass factors such as diffusion, membrane trafficking, and the complexities of cellular architecture.

**X. CONCLUSIONS**

The β-cell literature is formidably complex, exacerbated by limited communication between metabolic and electrophysiological investigators. This review has been an attempt to provide an “external” bioenergetic scrutiny of the field, to question some of the conventional approaches, and to highlight areas that have yet to attract significant attention. The hope is that experts in the field will appreciate that this relatively experienced mitochondrial physiologist has experienced in trying to extract a coherent cell biology thread from the literature.

In the absence of significant information on the isolated β-cell mitochondria, it is difficult to establish whether the organelle has evolved specific characteristics to function as the central transducer of glucose availability into adenine nucleotide phosphorylation potential. The mitochondria in liver, heart, brown adipose tissue, etc., each have distinctive characteristics suited to their in situ function. If mitochondrial transplantation were possible, would a β-cell containing muscle mitochondria still be functional in GSIS? The way in which the central metabolic/bioenergetic axis from glucose to insulin secretion responds to altered glucose availability should, in theory, be accessible to quantitative metabolic control analysis that could identify key control points for potential pharmacological intervention. The contentious role of coupling factor pathways might benefit from asking whether we are looking at a refilling of metabolite pools depleted by experimental starvation, rather than the production of unique signaling messengers. In general, investigations that correlate metabolic status with insulin secretion could perhaps place more emphasis on oscillatory phenomena at the plasma membrane. Finally, applying physiologically relevant conditions, be it in the context of \(\text{K}_{\text{ATP}}\) electrophysiology in relation to the cytosolic adenine nucleotide pool, or the unbound concentrations of fatty acids and derivatives, could restrict experimental degrees of freedom and facilitate their integration into the overall GSIS picture.

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**DISCLOSURES**

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**REFERENCES**


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