From The Rolf Luft Research Center for Diabetes and Endocrinology, Department of Molecular Medicine and Surgery, Karolinska Institutet, Karolinska University Hospital, Stockholm, Sweden

Temporal monitoring of intracellular Ca^{2+} signaling and origins of Ca^{2+} oscillations

Dominic-Luc Webb

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Abstract

This thesis examined parameters influencing stimulated cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) oscillations in hepatocytes and pancreatic β-cells. Hepatic glucose output is regulated in part by hormones such as vasopressin that act through $[Ca^{2+}]_i$ oscillations. Pulsatile $[Ca^{2+}]_i$ in β-cells parallels insulin secretion and this results in potently controlled blood glucose homeostasis. Employing temporal $[Ca^{2+}]_i$ measurements and related biochemical assays, efforts were made to understand how $[Ca^{2+}]_i$ transients are generated and maintained.

In studies of hepatocyte $[Ca^{2+}]_i$ signaling, we have demonstrated the existence of a phospholipase C (PLC) linked receptor driven Ca^{2+} entry pathway that, unlike capacitative Ca^{2+} entry, is independent of Ca^{2+} release from intracellular stores. These findings were challenged on the grounds that we used the cell permeant fluorescent Ca^{2+} indicator Fura-2 AM, which in some cases can be compartmentalized. The microinjection technique was established to rapidly introduce cell impermeant fura-2 into hepatocytes and subsequently monitor $[Ca^{2+}]_i$. Extracellular Mn^{2+}, which quenches fura-2 fluorescence upon entering the cell, was used as a Ca^{2+} surrogate. Following depletion of intracellular Ca^{2+} stores by thapsigargin or 2,5-di-(t-butyl)hydroquinone, vasopressin accelerated Mn^{2+} influx. Microinjected or loaded indicator yielded similar results. The source of Ca^{2+} was extracellular. Hepatocytes therefore possess at least one carrier for extracellular Ca^{2+} entry into the cytosol (i.e., non-capacitative) in addition to the capacitative Ca^{2+} entry pathway.

The methodology used above was extended to pancreatic β-cells to determine if the second messenger cyclic adenosine diphosphate ribose (cADPR) influences glucose induced $[Ca^{2+}]_i$ changes. Glucose was fully able to increase $[Ca^{2+}]_i$ whether fura-2 was microinjected alone, or together with cADPR or its competitive inhibitor $8NH_2$-cADPR. Similar results were obtained with carbamylcholine and KCl. cADPR did not alter insulin release at any $[Ca^{2+}]$ in permeabilized β-cells. A direct and rapid role for cADPR in normal insulin secretion was rejected.

The possibility that oscillatory insulin secretion is caused by glycolytic oscillations generated by muscle phosphofructokinase (PFK-M) was examined. According to this model, islet PFK-M experiences periodic bursts in activity via autocatalytic activation by its product, fructose 1,6-bisphosphate (F16BP). Primary pancreatic β-cells were treated with dihydroxyacetone (DHA), which enters glycolysis as the glycolytic intermediate dihydroxyacetone phosphate (DHAP). DHA was found to trigger $[Ca^{2+}]_i$ oscillations, most likely by increasing [F16BP] into the autocatalytic range at which PFK-M can oscillate. Substimulatory glucose (4 mM) was required in addition to DHA, indicating that glycolytic flux through PFK-M is obligatory. Attempts to measure [F16BP] in response to both glucose and DHA revealed large variability in this parameter. $[Ca^{2+}]_i$ oscillations tightly paralleled those of the ATP/ADP ratio.

Metabolic features of a transgenic mouse with a disruption in the promoter region of the *pfkm* gene were studied. This animal displayed tissue-specific loss of PFK-M functional expression with about 95% reduction in pancreatic islets. Glucose induced $[Ca^{2+}]_i$ oscillations were unaltered, but average amount and amplitude of insulin secretion were impaired. This might be explained by observations that maximum glucose utilization is about 100 times below PFK Vmax. This animal displayed impaired glucose tolerance *in vivo* without insulin resistance. The incomplete loss of PFK-M combined with impaired insulin secretion without impairment in $[Ca^{2+}]_i$ response suggested a minor impediment in glycolytic flux that is more detrimental to insulin exocytosis than $[Ca^{2+}]_i$ transients. No difference in the ATP/ADP ratio was detected.

Because fructose 2,6-bisphosphate (F26BP) can serve as a potent PFK-M activator, this was examined in β-cells. Microinjection of F26BP did not alter subsequent glucose induced $[Ca^{2+}]_i$ oscillations, consistent with published knowledge that the affinity of F26BP binding to PFK-M is reduced by the high intracellular citrate levels thought to exist in β-cells. An enzymatic assay established to measure [F26BP] in small numbers of islets indicated that [F26BP] remains at or below 3 μM in islets cultured at 11 mM glucose. Thus, at physiological glucose levels, [F26BP] is relatively constant in islets and cannot in any event affect $[Ca^{2+}]_i$ oscillations.

The hepatocyte study demonstrated that multiple Ca^{2+} entry pathways are activated by hormones known to trigger $[Ca^{2+}]_i$ oscillations. This could offer different permutations for driving and maintaining oscillations. This is likely applicable to other cell types. The pancreatic β-cell studies collectively show that glucose metabolism is a prerequisite for the slow $[Ca^{2+}]_i$ oscillations that parallel pulsatility in insulin release. Moreover, insulin secretion is more sensitive to reduced islet PFK-M expression than are increases in ATP/ADP ratio and oscillations in $[Ca^{2+}]_i$.

CONTENTS

LIST OF PUBLICATIONS

I Kass, G.E.N., **Webb, D.-L.,** Chow, S.C., Llopis, J. and Berggren, P.-O. Receptor-mediated Mn^{2+} influx in rat hepatocytes: comparison of cells loaded with Fura-2 ester and cells microinjected with Fura-2 salt. Biochem. J. 302 (Pt 1):5-9. 1994

II **Webb**, **D.-L.,** Islam, M.S., Efanov, A.M., Brown, G., Köhler, M., Larsson, O. and Berggren, P.-O. Insulin exocytosis and glucose-mediated increase in cytoplasmic free Ca^{2+} concentration in the pancreatic β-cell are independent of cyclic ADP-ribose. J. Biol. Chem. 271(32):19074-79. 1996

III Juntti-Berggren, L., **Webb, D.-L.,** Arkhammar, P.O.G., Schultz, V., Schweda, E.K.H., Tornheim, K. and Berggren, P.-O. Dihydroxyacetone-induced oscillations in cytoplasmic free Ca^{2+} and the ATP/ADP ratio in pancreatic β-cells at substimulatory glucose. J. Biol. Chem. 278(42):40710-16. 2003

IV Richard, A.-M.T., **Webb, D.-L.**, Goodman, J.M., Schultz, V., Flanagan, J.N., Getty-Kaushik, L., Deeney, J.T., Yaney, G.C., Dunaway, G.A., Berggren, P.-O. and Tornheim, K. Impaired insulin secretion in mice with tissue-dependent deficiency in phosphofructokinase-M. Submitted for publication

ABBREVIATIONS

AMP,ADP,ATP	adenosine 5' mono-, di- and tri- phosphate, respectively
cAMP	cyclic adenosine 3',5'-monophosphate
cADPR	cyclic adenosine 5´-diphosphate ribose
$8NH_2$-cADPR	8-amino cyclic adenosine 5´-diphosphate ribose, cADPR antagonist
$[Ca^{2+}]_i$	cytoplasmic free Ca^{2+} concentration
CCE	capacitative Ca^{2+} entry
CCh	carbamylcholine
CICR	Ca^{2+} induced Ca^{2+} release
CSIICR	Ca^{2+} sensitized IP_3 induced Ca^{2+} release
DHA	dihydroxyacetone (not a glycolytic intermediate, applied exogenously)
DHAP	dihydroxyacetone phosphate
DM	diabetes mellitus, all forms
DTPA	diethylenetriaminepentaacetic acid (manganese chelator)
F16BP	fructose 1,6-bisphosphate
F16Bpase	fructose 1,6-bisphosphatase (EC 3.1.3.11)
F26BP	fructose 2,6-bisphosphate
F26Bpase	fructose 2,6-bisphosphatase (EC 3.1.3.46)
F6P	fructose 6-phosphate
GA	d-glyceraldehyde (not a glycolytic intermediate)
IP_3	inositol 1,4,5-trisphosphate
K_{ATP}	ATP-sensitive K^+ (channel)
NAD[1]	β-nicotinamide adenine dinucleotide, oxidized form
NADH[1]	β-nicotinamide adenine dinucleotide, reduced form
NADP[1]	β-nicotinamide adenine dinucleotide phosphate, oxidized form
NADPH[1]	β-nicotinamide adenine dinucleotide phosphate, reduced form
NAD(P)H[1]	NADH and NADPH (autofluorescence, mainly mitochondrial NADH)
NCCE	non-capacitative Ca^{2+} entry
pCa	negative log of $[Ca^{2+}]$ (e.g., pCa 6 is 1 μM)
PFK	6-phosphofructo-1-kinase (EC 2.7.1.11)
PFK-M	6-phosphofructo-1-kinase (EC 2.7.1.11), muscle isoform
PFK-L	6-phosphofructo-1-kinase (EC 2.7.1.11), liver isoform
PFK-C	6-phosphofructo-1-kinase (EC 2.7.1.11), C-type subunit isoform
PFK2	6-phosphofructo-2-kinase (EC 2.7.1.105)
pH_i	intracellular pH
PLC	phospholipase C
T2DM	Type 2 diabetes mellitus
tBuHQ	2,5-di-(t-butyl)hydroquinone
Thaps	Thapsigargin

[1] Some older literature cited in this work abbreviate the pyridine nucleotides NAD, NADH, NADP and NADPH as DPN, DPNH, TPN and TPNH, respectively.

INTRODUCTION

Metabolic pulsatility as a global *in vivo* phenomenon

This thesis examines features of the pulsatile nature of cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in hepatocytes and pancreatic β-cells; two cell types of interest in diabetes mellitus (DM). In different cell types within different organs, very different outcomes result from the same basic input of increased $[Ca^{2+}]_i$. In addition to the initial increase in $[Ca^{2+}]_i$ evoked by extracellular signals, this cation typically displays oscillations, the particular waveform of which has physiological relevance. For example, changes in $[Ca^{2+}]_i$ oscillation frequency in lymphocytes have been shown to alter gene expression, this having important downstream functional consequences (1,2). All of the major cell types involved in metabolism and blood glucose homeostasis display $[Ca^{2+}]_i$ oscillations, including, but not limited to, glucagon and somatostatin secreting pancreatic islet cells (3), pancreatic β-cells (4), hepatocytes (5,6) and even cells of peripheral tissues, such as smooth (7) and skeletal (8) myocytes and hypothalamic neurons (9). A smaller amount of evidence exists for $[Ca^{2+}]_i$ (10) and metabolic (11) oscillations in adipocytes. Fluctuations in $[Ca^{2+}]_i$ are an integral component of a cell's response to changes in the extracellular environment. In normal *in vivo* physiology, there exists a highly synchronized multiple organ response to changes in nutrients such as glucose in the circulatory system that results in continuous homeostatic control of blood glucose concentration. It is a popular opinion that disturbances in insulin oscillations, which parallel $[Ca^{2+}]_i$ oscillations in pancreatic β-cells (12,13), are pathological (14) and represent an early event in the pathogenesis of type 2 DM (T2DM, 15,16). The most widely prescribed class of drugs issued to treat T2DM, the sulfonylureas, are known to modulate β-cell $[Ca^{2+}]_i$ oscillations (17,18).

Attempts to quantify *in vivo* metabolic parameters relevant to DM in humans with some degree of time resolution date back to at least the invention of the "diabetometer" in the mid 1800's (19,20 and pictured on the cover of this thesis). The 1923 doctoral thesis of Hansen (21) was devoted to temporal measurements of human blood glucose sampled at the ear lobe using a recently established ferricyanide based non-enzymatic assay for reducing sugars such as glucose (22). That series of studies clearly displayed several recordings of oscillating blood glucose with a period of several minutes in healthy as well as juvenile diabetic subjects and cited earlier works by other researchers who published similar results in 1920 (23). Thus, the landmark observation and confirmation that blood glucose oscillates both in healthy and juvenile diabetic humans date back to the first report of the discovery of insulin in 1922 (24), and established a foundation from which future studies of *in vivo* metabolic pulsatility are derived. Most "continuous" glucose measurements of the present time are intended for longer term monitoring (1-3 days) with typical sampling rates being around 1 sample per 20 minutes or less. This has revealed much slower oscillations (period around 110 minutes) in glucose as well as insulin (25) and also ultradian cyclicity in insulin (26). Much of this thesis deals with "slow" $[Ca^{2+}]_i$ oscillations of pancreatic β-cells that are believed to exist in parallel with the 4-10 minute *in vivo* glucose oscillations described by Hansen.

The establishment in the late 1950's of insulin radioimmunoassay (RIA, 27) made possible routine measurements of miniscule amounts of circulating insulin (28). Combining insulin RIA with glucose measurements, Anderson *et al* described in 1967 coordinated pulsatility in circulating glucose and insulin in dogs (29). Goodner *et al* demonstrated in 1977 synchronous oscillations in circulating insulin, glucagon and glucose in the rhesus monkey, and observed synchrony to a lesser extent in insulin and glucose in baboons and humans (30). Glucagon and insulin peaks were markedly out of phase, while insulin and glucose peaks were clearly in

phase. Consistent with what might have been inferred in the 1920's, Anderson *et al*, Goodner *et al* and others (25) proposed that insulin and glucagon oscillations follow, rather than lead, glucose oscillations. Under fasting conditions, it has been shown that hepatic glucose production oscillates in synchrony with islet hormones (31). Detailed descriptions of *in vivo* synchronous glucose, insulin and glucagon oscillations in humans were reported in 1983 (32,33). It was subsequently shown that less insulin is required to maintain glucose homeostasis when administered in pulses (34), this being explained by higher sustained levels of insulin receptors (35).

Insulin secreted from the pancreas enters blood circulation via the portal vein and is first seen by the liver. When insulin binds its receptor, it is internalized (36), thus removing it from the blood. About 50% of insulin secreted from the pancreas is immediately removed from circulation by the liver (37). The liver is obviously in the most privileged site in the entire body to experience and respond to pulsatile insulin secretion. Portal vein measurements (38) have demonstrated insulin oscillations with concentrations ranging from troughs at the RIA detection limit to peaks that are several orders of magnitude higher. Values at more peripheral sites show insulin oscillations with not more than 3-5 fold swings in concentration (39).

Metabolic pulsatility as an *ex vivo* single organ and single cell phenomenon

In response to changing ambient glucose concentrations, perifused rat pancreas adjusts the levels of pulsatile release of insulin, glucagon and somatostatin (40). Similarly, pulsatility in glucagon was shown to optimize glucose output in perifused liver (41).

Because this thesis examines signal transduction cascades involved in $[Ca^{2+}]_i$ oscillations, one very important concept is that of the second messenger, which has its origins in studies of liver metabolism. The second messenger bridges the gap between extracellular signals (e.g., hormones) and intracellular responses (e.g., $[Ca^{2+}]_i$ transients). This occurs via specific receptors in the plasma membrane. In 1957, the hepatocyte plasma membrane was shown to be required in order for the hormones adrenaline and glucagon (i.e., primary messengers) to evoke hepatocyte glycogenolysis, which was correlated with the formation of a compound similar to ATP or AMP (42). This compound was shown to be able to replace the hormones in cell extracts, and was quickly identified as cAMP (43). Such compounds are referred to as second messengers. This first description of a second messenger was followed by many others, most notably inositol 1,4,5-trisphosphate (IP_3) and diacylgrycerol (DAG)(44) formed by inositol 4,5-bisphosphate (PIP_2) breakdown by phospholipase C (PLC). Transient $[Ca^{2+}]_i$ increases in response to elevated IP_3 acting on specific receptors is now textbook knowledge.

With this background, Papers I-IV and some unpublished work can now be introduced sequentially. Paper I examined the temporal pattern of the hepatocyte $[Ca^{2+}]_i$ response during PLC activation. Paper II studied another second messenger, cyclic adenosine phosphate ribose (cADPR, 45) in pancreatic β-cells to gain insight regarding an intracellular Ca^{2+} pool that is insensitive to IP_3. Papers III and IV and the unpublished data in the remainder of this thesis examined a hypothesis that glycolytic oscillations driven by muscle phosphofructokinase in response to elevated glucose underlie the slow $[Ca^{2+}]_i$ oscillations in pancreatic β-cells that parallel insulin oscillations.

Paper I - Vasopressin, $[Ca^{2+}]_i$ oscillations and non-capacitative Ca^{2+} entry in the hepatocyte

In addition to insulin and glucagon, hepatocytes have receptors for, and are partly under the control of vasopressin, a 1 kD nonapeptide that originates from the hypothalamus (46-48, also called antidiuretic hormone). Isolated hepatocytes display $[Ca^{2+}]_i$ oscillations in response to a sustained increase in vasopressin (5), acting on G-protein coupled V1a receptors (49) that activate PLC, triggering glucose production (48,50-53). Unlike glucagon, vasopressin does not increase cAMP in hepatocytes (reviewed in ref. 54); the increased $[Ca^{2+}]_i$ can also trigger glucose production. Vasopressin evoked $[Ca^{2+}]_i$ oscillations were characterized as being frequency modulated (0.5-1.1 spikes/min) by receptor agonist concentration (5), although the positive feedback mechanism was unknown. From a broader perspective, one physiological role for PLC linked receptor activated $[Ca^{2+}]_i$ oscillations in the hepatocyte might be to acutely modulate glucose output.

It was known prior to the studies leading to Paper I that a rise in $[Ca^{2+}]_i$ evoked by PLC activation consists of more than just entry from intracellular stores and that the source of the additional $[Ca^{2+}]_i$ was extracellular (55). It became clear that elevated $[Ca^{2+}]_i$ triggers entry of extracellular Ca^{2+}. This is called capacitative Ca^{2+} entry (CCE), and according to the original model (56), following release of ER Ca^{2+} into the cytosol, elevated $[Ca^{2+}]_i$ itself activates Ca^{2+} entry from the extracellular milieu by activating store operated channels at the plasma membrane. CCE therefore represented a potential positive feedback mechanism for driving $[Ca^{2+}]_i$ oscillations, and for replenishing ER stores so as to maintain repetitive cycles of Ca^{2+} release. The CCE pathway appeared to be highly selective for Ca^{2+} over Mn^{2+} (57,58). In several different cell types, emptying of intracellular Ca^{2+} stores did not appear to be equivalent to PLC activation (59-63).

In addition to triggering an increase in $[Ca^{2+}]_i$ by release from intracellular stores, we described in a paper related to Paper I that in the hepatocyte vasopressin activates entry of extracellular Ca^{2+} by two pharmacologically separable, yet similar, "Ca^{2+} carriers" situated in the plasma membrane (64). One of these was permeable to Mn^{2+} and the existence of an entry pathway in addition to CCE in hepatocytes was proposed. This is referred to hereinafter as "non-capacitative Ca^{2+} entry" or NCCE. The existence of the NCCE pathway was challenged by the authors of the CCE pathway who argued that our observation of NCCE was an artifact of Fura-2 AM loading (65). Paper I of this thesis set out to further test the existence of the NCCE pathway. Methodology was established to combine microinjection and microfluorimetric measurements of $[Ca^{2+}]_i$ and Mn^{2+} (as a Ca^{2+} surrogate) influx in order to directly compare microinjected Fura-2 free acid with the cell permeant Fura-2 AM. Experiments were also performed to determine the intracellular fate of Fura-2 AM and Mn^{2+}.

Papers II-IV and unpublished findings - pancreatic β-cells

Ca^{2+} signaling within the context of glucose induced insulin secretion has been reviewed extensively (66,67). The basic working model for slow oscillations requires glucose to be taken up by GLUT transporters and metabolized, with glucokinase possessing a high degree of control over metabolic flux. ATP is formed at the expense of ADP, which increases the ATP/ADP ratio. The K_{ATP} channels respond to the increased ATP/ADP ratio by closing. These channels conduct an outward K^+ flux and are responsible for setting the plasma membrane potential, which at rest is about -70 mV. Upon closing in response to the elevated ATP/ADP ratio, there is a depolarization to around -40 mV, at which time voltage-gated Ca^{2+} channels

respond by opening and conducting an inward Ca^{2+} flux that raises $[Ca^{2+}]_i$. The elevated $[Ca^{2+}]_i$ then serves as a trigger to initiate and maintain insulin secretion through interactions with Ca^{2+} binding proteins (68-70). The $[Ca^{2+}]_i$ then declines by the combined actions of Ca^{2+} pumps (Ca^{2+}-ATPases) in the endoplasmic reticulum and plasma membranes, while Ca^{2+} and voltage activated K^+ channels (i.e., outward flux) facilitate repolarization and the K_{ATP} channels begin to re-open. At this point, several minutes have elapsed and one cycle of the slow $[Ca^{2+}]_i$ oscillations terminates. No source of positive feedback is specified in this basic model.

Superimposed on the peaks of these "slow oscillations" are "fast" oscillations with a frequency of multiple cycles per minute (71). While the slow $[Ca^{2+}]_i$ oscillations temporally parallel metabolic (ie., ATP/ADP ratio) and insulin secretion oscillations, the fast $[Ca^{2+}]_i$ oscillations appear to be driven by different (or additional) mechanisms, albeit coordinated with slow oscillations (72). At stimulating glucose concentrations, β-cells can display fast $[Ca^{2+}]_i$ oscillations in response to PLC-linked receptor agonists such as carbamycholine and vasopressin (73,74).

Paper II – cADPR and the pancreatic β-cell response to glucose

The existence of non-mitochondrial Ca^{2+} stores, in addition to those releasable by IP_3, were described many years ago (75,76). Due to its positive feedback characteristics, Ca^{2+} induced Ca^{2+} release (CICR) has been postulated as a mechanism for driving $[Ca^{2+}]_i$ oscillations. An initial $[Ca^{2+}]_i$ response to an agonist is amplified by Ca^{2+} itself triggering Ca^{2+} release from either Thaps and IP_3 (77) or caffeine and ryanodine (78) sensitive intracellular Ca^{2+} stores. When Paper II was initiated, CICR was thought to occur in pancreatic β-cells (79), but the importance of caffeine and ryanodine sensitive Ca^{2+} stores for glucose induced insulin secretion was less clear. It was widely understood that IP_3 generated from PLC agonists triggers $[Ca^{2+}]_i$ transients in this cell type (75,80,81).

Cyclic adenosine 5′-diphosphate ribose (cADPR) was identified as a novel second messenger that is synthesized from NAD by ADP ribosyl cyclase in sea urchins (45), and this enzyme activity is represented by the lymphocyte marker CD38 in mammals (82). Not long after its discovery, cADPR, acting through binding to ryanodine receptors (RyR), was proposed as a modulator of CICR in pancreatic β-cells (83). Interconversions between the different pyridine nucleotides were known to occur in islets in response to changing glucose concentration (84-86). It could have been hypothesized that such interconversions (e.g., cycling between NADH and NAD) might drive cADPR synthesis. Takasawa *et al* described Ca^{2+} release by cADPR, but not IP_3, in islet microsomes (87) and shortly after described a high abundance of CD38 in the pancreatic islet (88). However, the proposed mechanism of action (87,89) entirely bypassed the established role of β-cell electrical excitability in glucose induced insulin secretion (66). The cADPR model was further questioned by the inability of our group as well as others to reproduce this effect (90-92). The cADPR controversy was addressed in Paper II in the hopes of gaining a better understanding of IP_3-insensitive Ca^{2+} stores. Using microinjection of NAD, cADPR and the antagonist $8NH_2$-cADPR, as well as permeabilized pancreatic β-cells, Paper II examined a role for cADPR in glucose induced insulin secretion.

Paper III, IV and unpublished data - PFK-M driven $[Ca^{2+}]_i$ oscillations in β-cells

Much work has been published over many years regarding glycolytic oscillations driven by the third enzyme of glycolysis, 6-phosphofructo-1-kinase (EC 2.7.1.11, PFK) in yeast (93) and skeletal muscle (94), but this knowledge was not linked to oscillations in $[Ca^{2+}]_i$ and insulin

secretion of the pancreatic β-cell or T2DM until the late 1980's (95,96). The PFK reaction is irreversible, with a free energy for the forward reaction of -20 $kJ \cdot mol^{-1}$ (97) and is the first purely glycolytic step in metabolism. The reverse reaction is conducted by another protein, fructose 1,6-bisphosphatase (F16BPase), the liver isoform of which has only recently been described in pancreatic β-cells (98). Much of this thesis involved studies in pancreatic islets and β-cells of the muscle isoform of this enzyme (PFK-M). This isoenzyme is co-expressed in β-cells with two other mammalian subunit isoforms (99): liver (PFK-L) and a form found in most other tissues (PFK-C). The three isoforms are expressed from three separate genes on different chromosomes (100). PFK is active when it is in its tetrameric configuration and catalyzes phosphorylation of the number one carbon of fructose-6-phosphate (F6P), yielding fructose 1,6-bisphosphate (F16BP, ref. 101). PFK-M activity can be amplified (i.e., positive feedback) by binding of F16BP to a receptor on PFK-M distinct from the catalytic site. Both homo and hetero tetramers are possible (102) and both are active, although details of cooperativity between subunits are not fully understood. Figure 1 illustrates key components of the model for glucose induced metabolic oscillations that are proposed to underlie pulsatility in ATP/ADP ratio, $[Ca^{2+}]_i$ and insulin secretion. PFK-M is specified exclusively because only this isoform possesses the requisite regulatory properties to generate glycolytic oscillations and because it is thought to contribute the highest enzymatic activity (99). The positive feedback provided by F16BP is ultimately counter-acted by the combined actions of elevated citrate and ATP concentrations and loss of AMP, terminating one oscillatory cycle.

Glucose induced oscillations in insulin secretion correlate with those in ATP/ADP ratio, O_2 consumption and $[Ca^{2+}]_i$ (slow oscillations) with similar period (103,104). Descriptions exist of oscillations in NAD(P)H autofluorescence (105), lactate (106) and glucose uptake (107) that also show similar period and fit well with the PFK-M model. In addition to closing K_{ATP} channels, ATP/ADP ratio also contributes to insulin secretion (108) by altering phosphorylation levels of many proteins. PFK-M is the only PFK isoform expressed in skeletal muscle, and it has been shown to generate metabolic oscillations via positive feedback from F16BP in cell-free extracts (94,109); a feature only weakly possessed by the other isoforms, if at all in the intact cell (110,111). This indicates that PFK-M is capable of generating oscillations, as compared to merely following them.

Regulation of PFK-M is complex, involving protein-protein interactions with calmodulin (112) and microtubules (113), as well as phosphorylation and allosteric effects (114,115). PFK-M occupies a critical site in glycolysis at which a strong activity bottleneck (i.e., cross-over point) has long been known (116). In addition to F16BP, PFK-M can be activated by most other sugar bisphosphates (e.g., F26BP, G16BP, R15BP, etc) as well as AMP, ADP, phosphate, cAMP, elevated pH and others. It can be inhibited by citrate, lack of divalent cations such as Mg^{2+}, high concentrations of ATP (regulatory site) and acidic pH. Both activating (117-119) and inhibiting (120,121) actions of Ca^{2+} on PFK activity have been described. In the pancreatic β-cell, increased calmodulin concentration inhibits insulin secretion without apparent metabolic perturbation (122), and calmodulin inhibitors also inhibit insulin secretion (123). These effects have thus far been explained by actions of calmodulin on Ca^{2+} channel activity rather than PFK-M activity. Insulin secretion oscillations have been seen under conditions in which $[Ca^{2+}]_i$ is elevated but not oscillating (124). The model depicted in figure 1 describes metabolic oscillations driven by PFK-M that in turn drive $[Ca^{2+}]_i$ and other oscillations. The model does not exempt $[Ca^{2+}]_i$ or calmodulin feedback into PFK-M activity in synchrony with the oscillating allosteric regulators (e.g., F16BP, etc).

It was hypothesized that if F16BP could be artificially elevated in intact pancreatic β-cells at substimulatory glucose concentrations then PFK-M could be provoked into an oscillatory activity that would trigger $[Ca^{2+}]_i$ oscillations. Paper III tested this hypothesis by treatment of β-cells with exogenous dihydroxyacetone (DHA). It was reasoned that DHA would enter glycolysis at the aldolase/triose phosphate isomerase step as the glycolytic intermediate dihydroxyacetone phosphate (DHAP) following entry into the cell and phosphorylation by triokinase (125). Increased DHAP was predicted to increase F16BP and raise PFK-M activity into the autocatalytic range at low glucose, evoking downstream swings in ATP/ADP ratio and $[Ca^{2+}]_i$.

A further hypothesis was that a knockout of PFK-M by means of targeted gene disruption would result in loss of metabolic pulsatility. This was predicted to result in loss of oscillations in $[Ca^{2+}]_i$ and insulin secretion as well as perturbations in glucose homeostasis *in vivo*. A PFK-M deficient mouse was studied in which a disruption tag was inserted between the two known promoters of the *pfkm* gene. This animal showed sharply reduced expression of PFK-M in the pancreatic islet and was studied in Paper IV within the context of slow $[Ca^{2+}]_i$ oscillations and the corresponding insulin secretion oscillations. *In vivo* glucose tolerance and insulin resistance were also assessed in these animals.

As indicated in figure 1, there is a side reaction from F6P leading to F26BP by the enzyme 6-phosphofructo-2-kinase (PFK2, ref. 126). F26BP is not a glycolytic intermediate and PFK2 lacks a binding domain for positive feedback by F26BP, so PFK2 activity is not considered to be capable of oscillating. There is recent evidence that the PFK2 enzyme interacts directly with glucokinase and activates it, whereas F26BP gives no activation (127). At 130 nM, F26BP is capable of lowering the $S_{0.5}$ of F6P for PFK-M about fourfold, and is not a subtrate for aldolase or F16BPase (128). We reasoned that μM concentrations of F26BP might potently stabilize PFK-M into an elevated, non-oscillating activity. Published measurements of [F26BP] in pancreatic islets have yielded conflicting results. In freshly prepared islets in which glucose was not present during the islet isolation, glucose was found to dose-dependently increase [F26BP] (129). Subsequent studies using more physiological glucose concentrations prior to the experiment demonstrated no increase (130). In this thesis, unpublished recordings of glucose induced $[Ca^{2+}]_i$ oscillations in dispersed *ob/ob* β-cells microinjected with F26BP are presented as well as measurements of [F26BP] in *ob/ob* islets cultured at 11 mM glucose.

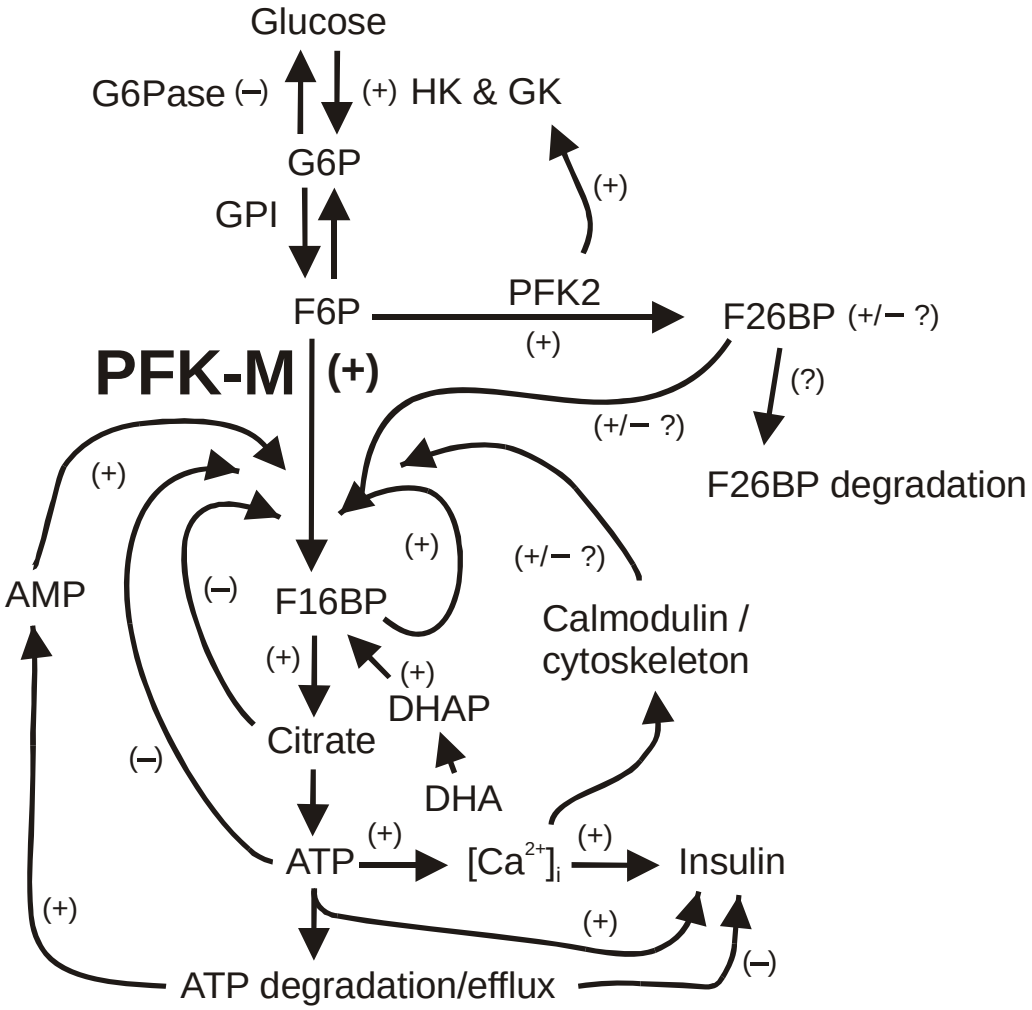

Fig. 1. Model of muscle phosphofructokinase (PFK-M) driven oscillations in ATP/ADP, cytoplasmic free Ca^{2+} ($[Ca^{2+}]_i$) and insulin secretion in the pancreatic β-cell. Glucose is metabolized to fructose 6-phosphate (F6P) via hexokinase (HK) and glucokinase (GK) and isomerization by glucose phosphate isomerase (GPI). PFK-M phosphorylates F6P, yielding fructose 1,6-bisphosphate (F16BP). Some F16BP binds the autocatalytic domain of PFK-M, causing a burst in PFK-M activity. Most F16BP carbons ultimately enter mitochondrial metabolism as pyruvate to form ATP, thus increasing ATP/ADP, Ca^{2+} influx and insulin secretion. Increased ATP (from glycolysis or mitochondrial metabolism) and citrate are powerful inhibitors of PFK-M, leading to a downswing in glycolytic flux. As ATP is consumed, PFK-M is re-activated by 1) increased levels of AMP and ADP relative to ATP and 2) reduction of inhibitory ATP binding, thus completing one oscillatory cycle. Exogenous dihydroxyacetone (DHA) can enter metabolism at the aldolase step after triokinase phosphorylation, raising [F16BP]. The side reaction of PFK2 phosphorylates F6P to fructose 2,6-bisphosphate (F26BP). Microinjected F26BP could potentially lead to loss of oscillatory activity by binding to the F16BP autocatalytic site of PFK-M, thus clamping enzyme activity to a more constant rate. The role of endogenous F26BP in islets is controversial, whereas PFK2 has been shown to enhance GK activity by a direct protein-protein interaction. Both activating (through calmodulin) and inhibiting (through cytoskeleton) actions of $[Ca^{2+}]_i$ on PFK-M activity have been described in other systems. This thesis examined the influence of DHA (Paper III), microinjected F26BP (unpublished) and PFK-M deficiency (Paper IV) on metabolic and $[Ca^{2+}]_i$ oscillations.

AIMS OF THE STUDY

The overall aim of this thesis is to understand the mechanisms underlying $[Ca^{2+}]_i$ oscillations pertaining to hepatocytes and pancreatic β-cells. The specific aims are as follows:

Paper I To further explore the existence of NCCE in the hepatocyte and determine whether loaded Fura-2 AM and microinjected Fura-2 yield different results with respect to Mn^{2+} entry in response to vasopressin.

Paper II To determine if cADPR under normal conditions is involved in glucose induced increases in $[Ca^{2+}]_i$ and insulin secretion in the pancreatic β-cell.

Paper III To determine if DHA, entering glycolysis downstream of PFK-M, can evoke $[Ca^{2+}]_i$ oscillations as a test of PFK-M to drive metabolic and other oscillations. DHA was predicted to elevate [F16BP] to a level at which PFK-M can oscillate at substimulatory glucose concentration.

Paper IV To identify changes in $[Ca^{2+}]_i$ and insulin oscillations in islet cells as well as *in vivo* glucose tolerance and insulin levels in a mouse with a disruption in the promoter region of the *pfkm* gene.

Unpublished (F26BP in the pancreatic β-cell) To determine if slow $[Ca^{2+}]_i$ oscillations in pancreatic β-cells are dampened by microinjected F26BP through allosteric effects on PFK-M and to measure F26BP concentration in these cells at physiologically relevant glucose concentrations.

METHODS

Animal models

Wistar rat - CCE had already been described in Wistar rat hepatocytes (131), so this particular model was used to study NCCE in Paper I. It was results from our previous studies of Wistar hepatocytes that first revealed an additional entry pathway (64). The first demonstration of vasopressin induced $[Ca^{2+}]_i$ oscillations also used rat hepatocytes **(**5). Further tests of the existence of NCCE pathway were desired without introducing uncertainties regarding cell type, species or other variables.

ob/ob mouse - Much of the work presented in this thesis used pancreatic β-cells from a local colony of *ob/ob* mice, which are described in detail elsewhere (132-136). Islets were in all cases isolated from mice between 10 and 12 months of age.

PFK-M deficient mouse - Heterozygous mice with a disrupting tag inserted in the noncoding region of the PFK-M gene (OmniBank OST#56064, 50% C57BL/6 albino, 50% 129svEvBrd) were from Lexicon Genetics Incorporated and were housed and bred at Boston University Medical Center. Wildtype and homozygous PFK-M (tentatively) knockout animals were bred exclusively from the heterozygous females because homozygous females displayed hyperglycemia.

Chemicals

The excitation and emission peaks (in nm) for the fluorescent indicators used are as follows: Fura-2 (Ex. 340/380, Em. 510); rhod123 (Ex. 490, Em.529); BCECF (Ex. 490, Em. 510); NAD(P)H (autofluorescence, Ex. 366, Em. 460).

The culture media for all mouse excised islet material was RPMI-1640 containing 11 mM glucose. Cell death in culture is minimal at this glucose concentration (137).

2,5-di-(t-butyl)hydroquinone (tBuHQ) and thapsigargin (Thaps) were used in Paper I to deplete intracellular Ca^{2+} stores. Both compounds, which are structurally dissimilar, are thought to act by blocking the refilling of these pools by inhibiting ER Ca^{2+}-ATPases. Saponin and nonidet P-40 are chemically similar biomembrane dissolving detergents, but have different efficacies for different types of membranes. This difference was exploited in Paper I. The plasma membrane was first permeabilized by saponin. This left IP_3 sensitive and other intracellular compartments intact. Intracellular membranes could subsequently be attacked by addition of nonidet NP-40. Ionomycin, also used in Paper I, is an antibiotic that acts by a carrier/transporter type mechanism to selectively transport Ca^{2+} and Mn^{2+} across phospholipid bilayers.

Diethylenetriaminepentaacetic acid (DTPA) was used in Mn^{2+} quenching experiments in Paper I. This chelator of multivalent cations was selected due to its higher affinity for Mn^{2+} and lower affinity for Ca^{2+} compared to EGTA or EDTA (138). The selectivity of DTPA for the ions of interest in the buffer is $Mn^{2+} > Ca^{2+} > Mg^{2+}$.

Procedures

Microinjection - Cell preparation and microinjection of Fura-2, NAD, cADPR and $8NH_2$-cADPR is described in detail in Papers I and II. Regarding F26BP microinjection experiments

(*ob/ob* β-cells), the target intracellular concentration was 1 μM or higher, and three concentrations were tested. F26BP was prepared as (nominally) 1, 10 or 50 mM stock solutions in the microinjection buffer (pH 7.0) indicated in Paper II. From these, aliquots were added directly into the tubes of Fura-2 pentapotassium salt from the vendor, yielding 25 mM of the dye, a 250-fold concentration above the estimated intracellular concentration of loaded Fura-2 AM. Because F26BP is acid labile, the pH of the F26BP-containing injection buffer was confirmed with pH paper prior to experiments. After correcting for purity and other losses (see results of F26BP assays), microinjection was estimated to increase the intracellular [F26BP] by 1, 10 or 50 μM, respectively. This protocol ensured that if a strong Fura-2 signal was registered then the F26BP was at or above the target intracellular concentration (see methods in Papers I and II).

Two parameters were used to calculate the effects of injected F26BP on the glucose response. First, the 340/380 fluorescence ratio of the initial glucose responses were evaluated and ranked qualitatively into 4 groups, and second, presence and amplitude of oscillations were similarly ranked. Because the experimental strategy aimed to potently stabilize PFK-M pulsatility, dramatic effects on $[Ca^{2+}]_i$ oscillations (e.g., a sustained plateau) were anticipated. Ranking of responses and oscillations were as follows: strong (4 points), medium (3 points, easily detectable), weak (2 points, present, but barely detectable), or no response/oscillation (1 point). All recordings were plotted on the same scale, and were assessed separately by two people. The values for the three groups (Fura-2 AM loaded, injected controls and F26BP injected) were compared with Students t-test.

Photomultiplier photometry - In all microfluorimetry experiments in papers I and II and some experiments in paper III, a photomultiplier tube (PMT) was used. These recordings were made one object (i.e., one cell or aggregate of connected cells) at a time. In some of the later studies, a charge-coupled device (CCD) camera was used, which offered spatial resolution.

CCD imaging of $[Ca^{2+}]_i$ - Imaging was performed with a peltier/ethylene glycol cooled CCD camera (Metachrome II, Photometrics Inc, USA). Before experiments using Fura-2, the two monochromators were adjusted at 360 nm excitation so as to equilibrate the intensities of the two beams. The excitation wavelengths were then set to 340 and 380 nm. In order to accurately subtract light-derived background noise, an empty, unoccupied region near the recorded cells within the CCD field was also recorded. This background noise (typically 2-5 counts) was subtracted. In studies using microinjection, recordings were also made on Fura-2 AM loaded cells in order to ensure that injected Fura-2 reached the same concentration as would be found in loaded cells. This was done to verify that microinjection had deposited the desired final concentration of injected test compounds. In aggregates with microinjected cells, uninjected cells were also selected for recording as a measure of cellular autofluorescence. This contributed no more than 1-2 counts and was negligible in these experiments. Some work in paper III, essentially all of paper IV, and all F26BP microinjection experiments used this CCD imaging system.

Insulin radioimmunoassay (RIA) - Measurements of insulin were performed exclusively by RIA, essentially as originally described in the literature (27).

ATP/ADP ratio - Bioluminometric measurements of islet ATP/ADP ratio were performed in Papers III and IV essentially as described (139). There are four major steps in this assay: 1) measurement of [ATP] in one aliquot, 2) conversion of ATP to AMP in a second aliquot; 3) conversion in the second aliquot of ADP to ATP; 4) measurement of [ATP] in second aliquot.

Step 1 yielded [ATP] and step 4 yielded [ADP]. Samples from perchloric acid deproteinization, as shown in the original paper (139), were not stable for more than a day, so a procedure utilizing trichloroacetic acid followed by ether extraction and lyophilization was adopted. Due to loss of sample volume during ether extraction, there were losses in ATP and ADP of equal but unknown magnitude. Absolute values for [ATP] and [ADP] would have been underestimations, but ATP/ADP ratios could be accurately reported. Due to limited amounts of tissue, ATP/ADP ratios in Paper IV were only measured for 1 and 5 minutes of glucose stimulation.

F16BP content - Enzymatic measurements of [F16BP] (Paper III) in islet extracts would not work in the presence of a high background of DHA. Two approaches were attempted to separate the low amount of endogenous F16BP from the comparatively high amount of applied DHA: 1) anion exchange HPLC using an ammonium formate gradient and determination of [F16BP] by electro-spray ionization mass spectrometry (ESI-MS) or an enzymatic cycling assay (140,141); and 2) anion exchange directly in a pipette tip followed by the cycling assay. The second approach proved more feasible and was used for this study. The enzyme assay coupled NADH from reacting F16BP, aldolase, triose phosphate isomerase and glyceraldehyde 3-phosphate dehydrogenase with cycling between malate and alcohol dehydrogenases in the presence of oxaloacetate and ethanol. Accumulated malate from this reaction was used as substrate for malic enzyme in the presence of NADP. The NADPH formed by this reaction was measured spectrophotometrically at 340 nm, which reflected F16BP content.

F26BP content, standards - An F26BP standard was prepared by acid hydrolysis of a small aliquot of a stock solution. Commercial F26BP powder was dissolved in 20 mM Tris acetate pH 7.8 to make a stock solution. This F26BP stock was mixed with 0.1 N HCl and hydrolysis was run at room temperature. After 10 minutes, HK buffer was added (100 mM triethanolamine and 10 mM $MgCl_2$, pH 7.6) and the reaction was neutralized with 0.1 N NaOH. As a control, another sample was run without acid hydrolysis. To each of these, NADP (10 mg/ml) and glucose 6-phosphate dehydrogenase (200 µg/ml) were added. A baseline was established and the samples were assayed for [F6P] by following the increase in NADPH absorption at 340 nm upon addition of phosphoglucose isomerase (1 mg/ml). Spectrophotometry was done with a Beckman DU7500 spectrophotometer equipped with the autosampling transport accessory and a 4 compartment quartz cuvette, each compartment holding a 300 µl volume.

F26BP content, microassay - The above assay for standardizing stock solutions was suitable for larger volumes and concentrations of F26BP. Measurements of smaller samples, such as islets from single animals, were carried out in a separate microassay which was essentially the same as that described by Van Schaftingen *et al* (142,143) using the potato pyrophosphate-dependent PFK (PPi-PFK), the velocity of which is dependent on [F26BP]. Two control parameters were appended: a) heat; and b) acid treatment. F26BP is known to be heat stable and acid labile. Recordings were done with the Beckman spectrophotometer using kinetics/time mode at 340 nm with background subtraction at 400 nm, following an earlier observation of a 340 nm background noise that has comparable absorbance at 340 and 400 nm. Data are shown as mean values of 4 sets of recordings for H_2O blanks and 3 mg/ml NADH in H_2O. A molar extinction coefficient of 6.2 $mM^{-1} \cdot cm^{-1}$ was used to convert absorbance values to [NADH], which was consumed in the reaction as a function of [F26BP]. The rate constants obtained were used to determine cellular [F26BP] against standards. Small aliquots of islets were set aside for $[Ca^{2+}]_i$ recordings to determine if the preparations used for [F26BP] measurements possessed glucose induced $[Ca^{2+}]_i$ oscillations. Preparations were separated into two groups depending on

presence or absence of $[Ca^{2+}]_i$ oscillations. Assuming F26BP is largely cytosolic, the estimated nuclear volume of the β-cell (approx. 400 fl) was subtracted.

The nominally 50 mM stock solution of commercial F26BP used for microinjection experiments was also assayed. Specifically, "nominally 50 mM" means F26BP was prepared from commercial powder according to a molecular weight of 428 assuming 100% purity and no water content. This was also tested with and without heat and acid treatment.

RESULTS

Results of Paper I: Non-capacitive Ca^{2+} entry in hepatocytes

Distribution and intensity of the Fura-2 fluorescence in hepatocytes by loading of the ester form or microinjection of the free acid appeared identical. It was possible to successfully inject several cells within a few seconds without any apparent morphological changes and without formation of blebs even a considerable time after recordings. Recovery from injection was rapid. Within 20 seconds following microinjection of Fura-2, single cell measurements yielded a baseline $[Ca^{2+}]_i$ comparable to Fura-2 AM loaded cells. Having established that the microinjection technique could be used to immediately monitor Fura-2 fluorescence so as to eliminate potential complications arising from compartmentalization of the loaded indicator, the effects of vasopressin in Fura-2 AM loaded versus Fura-2 microinjected cells were compared. By either of these methods, vasopressin, but not tBuHQ, increased the rate of Fura-2 quenching resulting from entry of extracellular Mn^{2+}. Neither the prior depletion of intracellular Ca^{2+} stores by tBuHQ nor the blockade of refilling of these stores by Thaps prevented the enhanced Mn^{2+} quenching evoked by vasopressin. The release of Ca^{2+} from intracellular stores was insufficient to elevate Mn^{2+} entry, whereas vasopressin receptor activation was required.

It was reasoned that sequestration of either Mn^{2+} or Fura-2 into intracellular stores could complicate the interpretation of these results. The experiments shown in figure 3 of Paper I address this issue. Only a small quench could be seen upon removal of extracellular Mn^{2+} by the selective chelator DTPA (Paper I, figure 3a), whereas a large quench was seen without DTPA (Paper I, figure 3b). Hence, the majority of the quench resulted from entry of Mn^{2+} from outside the cell and not by re-release of Mn^{2+} from intracellular stores. Permeabilization of the plasma membrane by the detergent saponin led to the loss of the overwhelming majority of Fura-2, a protocol which left intracellular stores intact. Only a small further loss of signal resulted from addition of the harsher detergent nonidet P-40, indicating only a minor accumulation of Fura-2 AM into intracellular stores.

Results of Paper II: Glucose induced insulin secretion is not dependent on cADPR

Experiments with microsomes and microinjection in sea urchin eggs were originally used to demonstrate the Ca^{2+} mobilizing effect of cADPR (144). This lab previously demonstrated that cADPR added to dispersed and permeabilized *ob/ob* islets does not evoke Ca^{2+} mobilization (91), directly challenging the findings of Takasawa *et al* (87). Microinjection of the precursor NAD into *ob/ob* β-cells failed to elevate $[Ca^{2+}]_i$ while CCh treatment gave a typical response (Paper II, figure 1). Subsequent elevation of glucose gave a rise in $[Ca^{2+}]_i$ characteristic of untreated cells and clearly established the cells that were recorded as pancreatic β-cells. KCl treatment after the glucose treatment further demonstrated an apparently normal influx of extracellular Ca^{2+} and capacity of the plasma membrane to repolarize and lower $[Ca^{2+}]_i$ back to a basal level.

Microinjection of $8NH_2$-cADPR, a potent antagonist of cADPR, did not alter the glucose induced rise in $[Ca^{2+}]_i$ (Paper II, figure 2). Not even a partial blockade was seen in the glucose response or in transients evoked by other agonists, such as CCh and KCl. In a separate series of experiments, microinjection of cADPR did not alter subsequent $[Ca^{2+}]_i$ responses to CCh, glucose or KCl (Paper II, figure 3).

The possibility that cADPR might function independently from changes in $[Ca^{2+}]_i$ reminiscent of cAMP (145) was also tested. Due to limited material, $8NH_2$-cADPR could not be used in insulin secretion experiments. ADPR was instead used as a specificity control in the event that cADPR altered insulin secretion. In permeabilized cells in which the cytosol was readily accessible by small molecules, insulin secretion was unchanged at any $[Ca^{2+}]$ of physiological relevance (i.e., pCa 8 to 4) by addition of either cADPR or ADPR (Paper II, figure 4).

Results of Paper III: DHA triggers $[Ca^{2+}]_i$ oscillations

Studies of the effects of glucose, GA, DHA on the intial increase $[Ca^{2+}]_i$ demonstrated that glucose consistently yielded a sustained increase in $[Ca^{2+}]_i$ while GA did so less frequently and DHA treatment only gave an initial increase (Paper III, figure 1). In those cases where a response was obtained, the initial rise appeared similar, compatible with a common mode of action. In longer recordings to study $[Ca^{2+}]_i$ oscillations, when DHA was given in the absence or presence of 4 mM glucose, $[Ca^{2+}]_i$ oscillations appeared only in the presence of the substimulatory glucose concentration (Paper III, figure 2). When 20 mM DHA was compared to 10 mM DHA + 4 mM glucose in order to equilibrate for the difference in carbons between the sugars (Paper III, figure 3), substimulatory glucose was still required to achieve oscillations.

To determine if DHA triggered $[Ca^{2+}]_i$ oscillations correlate with metabolic events, as by the proposed PFK-M driven mechanism, oscillations in ATP/ADP ratio and $[Ca^{2+}]_i$ in response to 10 mM DHA were measured for the same time interval. In response to DHA at substimulatory glucose concentration, ATP/ADP oscillations were seen to be highly synchronized with $[Ca^{2+}]_i$ oscillations (Paper III, figure 4). The stable baseline of the $[Ca^{2+}]_i$ trace indicated that these oscillations were not present until triggered by the DHA treatment.

The effect of DHA on insulin secretion was also examined in the presence or absence of glucose (Paper III, figure 5). DHA alone triggered only a weak insulin release while a far more potent release was seen when 4 mM glucose was also present. Glucose concentration was then raised to 11 mM. The group previously without glucose gave much more insulin secretion than the group that earlier received 4 mM glucose. Glucose, at 11 mM, was clearly the most potent secretagogue, although much of the first phase secretion was lost in the group initially treated with 4 mM glucose and 10 mM DHA.

Simultaneously recording the effects of glucose, DHA and GA on $[Ca^{2+}]_i$ and mitochondrial membrane potential revealed that glucose and GA had similar hyperpolarizing effects and time until onset of $[Ca^{2+}]_i$ increase (Paper III, figure 6). DHA, on the other hand, did not give potent hyperpolarization and when a rise in $[Ca^{2+}]_i$ occurred, this was greatly delayed compared to glucose and GA. Effects of 11 mM glucose, 10 mM DHA, and 10 mM GA on NAD(P)H levels were evaluated. Only glucose significantly elevated NAD(P)H (Paper III, figure 7), indicating that as fuels, GA and DHA are not equivalent to glucose.

The effects of 11 mM glucose, 10 mM DHA, 10 mM GA on pH_i also differed. Despite the comparatively weak effect of GA on mitochondrial membrane potential and NAD(P)H, GA produced the largest drop in pH, which, unlike glucose and DHA, was both rapid and sustained (Paper III, figure 8).

Results of Paper IV: Disruption of *pfkm* distal promoter perturbs glucose homeostasis

The introduction section of Paper IV describes previous knowledge regarding tissue specificity of the two promoter system of PFK-M. The degree of loss of PFK-M expression in the PFK-M deficient mice was quantified by Western blot in skeletal muscle, heart, brain and islet (Paper IV, figure 1). In brain, heart and skeletal muscle, there was a close agreement between changes in transcription levels and relative utilization of the distal versus proximal promoters (Paper IV, table 1). Islet transcription levels were not determined, but it was clear that the 95-98% loss of PFK-M expression in islets had resulted from the disruption near the distal promoter, demonstrating an overwhelming dependency of islet PFK-M expression on this promoter.

Paper IV, figure 2 shows glucose intolerance in the PFK-M deficient mice that was not accompanied by insulin resistance (Paper IV, figure 3). Hence, the glucose intolerance could be explained by defective insulin secretion. On average, basal insulin was somewhat higher than in control mice, but did not reach statistical significance. The fold increase in blood insulin following glucose injection was lower in the PFK-M deficient mice. Both fasted and non-fasted PFK-M deficient mice displayed higher glucose levels than control mice.

In perifusion experiments with isolated islets, insulin secretion (Paper IV, figure 4) was dramatically dampened and oscillations were more irregular in the PFK-M deficient group. No consistent difference was detectable in basal insulin secretion.

It was reasoned that a serious impairment in PFK activity, and by extension, metabolism, would lead to a lag in glucose stimulated ATP production that would delay K_{ATP} channel closure, and/or yield lower amplitude in ATP/ADP ratio changes that would result in reduced depolarization and $[Ca^{2+}]_i$ entry. However, examination of $[Ca^{2+}]_i$ oscillations in both cell clusters and single cells showed no detectable difference (Paper IV, figure 5). Basal $[Ca^{2+}]_i$, time of onset, initial $[Ca^{2+}]_i$ rise and subsequent "2nd phase" of response to elevated glucose were all similar between the wild type and PFK-M deficient animals. No pattern was seen in any of the data to suggest that a higher number of samples (i.e., more recordings) would have yielded a statistical difference. No detectable difference was found for glucose elevated ATP/ADP ratio.

As shown previously in rat islets (124), insulin oscillations were still present after clamping K_{ATP} channels open and raising $[Ca^{2+}]_i$ by combined treatment of diazoxide and KCl (Paper IV, figure 6).

Results of F26BP study - β-cell $[Ca^{2+}]_i$ oscillations are not modulated by F26BP

It was hypothesized that increasing [F26BP] in β-cells by microinjection might dampen glucose-induced $[Ca^{2+}]_i$ oscillations to a stable plateau by potently clamping PFK-M activity at an elevated level and inhibiting F16BP binding. Table 1 shows the tabulated data for the 8 mM glucose responses of cell aggregates loaded with Fura-2 AM, Fura-2 injected controls or cells microinjected with Fura-2 plus F26BP. The [F26BP] shown are corrected for purity, etc (see below). Microinjection reduced the frequency of occurrence of the oscillations as compared to Fura-2 AM loaded controls. However, there was no significant difference between the F26BP injected versus control injected groups. The qualitative scoring method used here was intended to identify dramatic dampening effects on slow $[Ca^{2+}]_i$ oscillations, as might be expected if the oscillator (i.e., PFK-M) were clamped at a stable plateau.

In order to make proper assessment of the microinjection data, it was necessary to know how much the intracellular [F26BP] was increased by microinjection. Because of the conflicting findings in the literature (129,130), the endogenous [F26BP] was not known with certainty. The [F26BP] in the *ob/ob* β-cell was therefore assayed under the conditions in which $[Ca^{2+}]_i$ measurements were made in the F26BP microinjection experiments. Results using the F26BP microassay for two islet preparations and microinjection stock solution are shown in Table 2. In both islet preparations, cytosolic [F26BP] was at or below 3 μM after 48 hours of culture at 11 mM glucose and 45 minutes incubation at 3 mM glucose, which was the protocol for the microinjection experiments. In the bulk assay to determine purity of commercial F26BP powder, the nominally 50 mM stock solution was found to be 60% pure. After the F26BP microinjection experiments were concluded, it was determined that roughly 50% of the originally calculated [F26BP] for the microinjection experiments had actually been injected into the cells. Correcting for these factors, the three pipette concentrations of F26BP were 0.5, 5.0 and 25.0 mM, yielding increases in intracellular [F26BP] of approximately 1, 10 and 50 μM, respectively.

Table 1

Effect of microinjected F26BP on glucose induced $[Ca^{2+}]_i$ oscillations. Shown are mean of response scores, standard error of mean (s.e.m.) and sample size (n) of initial response to increasing glucose from 3 to 8 mM and slow oscillations. In this set of experiments, the initial responses and oscillations of both microinjection groups yielded somewhat lower scores than the Fura-2 AM loaded groups. Microinjection with pipette concentrations of 0.5, 5.0 or 25 mM F26BP (corrected for purity, etc) did not reveal a difference between the control and F26BP injected groups.

	Initial glucose responses			Oscillations		
	loaded	**ctrl inj.**	**f26bp inj.**	**loaded**	**ctrl inj.**	**f26bp inj.**
0.5 mM F26BP*						
mean	3.8	3.0	3.3	2.8	2.5	2.4
s.e.m.	0.3	1.0	0.5	0.8	1.5	0.8
n	4	4	6	4	4	6
5 mM F26BP*						
mean	3.3	2.2	2.2	1.8	1.4	1.3
s.e.m.	0.3	0.3	0.3	0.2	0.2	0.1
n	21	14	25	21	14	25
25 mM F26BP*						
mean	3.4	2.2	2.8	2.9	1.8	2.0
s.e.m.	0.3	0.2	0.2	0.3	0.2	0.3
n	8	14	15	8	14	15

Response scores assigned as follows:
1.0 = no response
2.0 = weak response
3.0 = medium response
4.0 = strong response
Scoring of oscillations was based on amplitude, which would decrease if the oscillator was dampened. No change in frequency was seen.

* Intracellular [F26BP] was estimated to increase by 1, 10 and 50 μM following injection using pipette concentrations of 0.5, 5.0 and 25 mM F26BP, respectively

Table 2

F26BP concentration in *ob/ob* β-cell aggregates and microinjection stock solution. For determination of endogenous F26BP concentration in *ob/ob* β-cells, samples were cultured 48 hours at 11 mM glucose followed by 45 minutes incubation at 3 mM glucose. Presence or absence of glucose induced $[Ca^{2+}]_i$ oscillations was determined in a separate experiment. Both preparations measured by the microassay method responded to glucose. The "nominally" 50 mM F26BP microinjection stock solution was assayed at the conclusion of those experiments. The commercial powder was found in a separate "bulk" assay to be 60% pure, meaning that the freshly prepared microinjection stock solution of F26BP was actually 30 mM. The assayed value of 25 mM for the stock solution found after experiments were completed implies that a small amount of degradation also occurred. The pipette concentrations, corrected for these impurities and losses, for microinjection experiments in Table 1 are therefore 0.5, 5.0 and 25 mM, yielding increases in intracellular [F26BP] of approximately 1, 10 and 50 μM, respectively. No signal was detected after acid hydrolysis, consistent with the physical properties of F26BP.

	[F26BP]		
	+ acid	- acid	Oscillations
Experiment 1	0	3.0 μM	no
Experiment 2	0	1.0 μM	yes
Nominally 50 mM F26BP stock	0	25 mM	

DISCUSSION

Discussion of Paper I

The existence of NCCE in hepatocytes had been challenged on the grounds that under some conditions Fura-2 AM was found to sequester into intracellular Ca^{2+} stores (65). The microinjection and dye/Mn^{2+} sequestration results clearly speak against such an artifact. It was not the goal of paper I to refute the existence of the CCE pathway as originally described (56), but to further test the existence of an additional pathway. Figure 1 in Paper I might appear to refute the existence of CCE. It should be noted that the buffer used in these experiments did not contain Ca^{2+} and we previously demonstrated that hepatocyte CCE is not very permeant to Mn^{2+} (64). It was concluded that vasopressin induced Mn^{2+} entry is more easily explained by two separate plasma membrane carriers (i.e., channels). Use of channel antagonists did not support two independent conformations of a single channel (i.e., interconversion between Ca^{2+} and Ca^{2+}/Mn^{2+} permeable states) as has been described for other types of channels (146). TPCK and neomycin blocked part of the vasopressin induced Ca^{2+} influx without affecting Mn^{2+} influx. Both carrier systems were apparently active during the same time interval and are therefore not necessarily mutually exclusive.

Subsequent to publication of Paper I, a review by the same group that originally challenged NCCE described a model incorporating both NCCE and CCE pathways (147). This has the two pathways operating in the same cell, as supported in this thesis, but they are mutually exclusive. Figure 2 depicts a similar model, in which the two Ca^{2+} entry pathways are independent (i.e., two separate channels) but not mutually exclusive (i.e., both can operate simultaneously), in compliance with the findings of Paper I and our earlier study (64). It is thought that the NCCE pathway is responsive to IP_3, DAG and AA (148-150) rather than solely to elevated $[Ca^{2+}]_i$. Downstream events thought to link $[Ca^{2+}]_i$ transients to glucose production by interactions with calmodulin and tubulin are also shown in Figure 2.

A model for driving hepatocyte $[Ca^{2+}]_i$ oscillations called "Ca^{2+} sensitized IP_3 induced Ca^{2+} release" (CSIICR) has been proposed (151). According to this suggestion, regulation of the IP_3 response involves interactions of Ca^{2+} with one or both of two binding sites on the IP_3 receptor. The two sites are in close proximity to the channel pore and have very different binding affinities for Ca^{2+}. Because the channel is regulated by cAMP/PKA phosphorylation (152), CSIICR could be influenced by hormones that raise cAMP levels. Hepatocyte $[Ca^{2+}]_i$ transients generated by receptor agonists acting on intracellular Ca^{2+} stores through PLC have been shown to be potentiated by nM glucagon concentrations (153).

It has long been proposed that the physiological relevance of hepatocyte $[Ca^{2+}]_i$ oscillations would be to give sustained calmodulin activity while escaping the cytotoxic effects of sustained elevated $[Ca^{2+}]_i$ (154). Within physiological concentrations, vasopressin dose-dependently increases the frequency of hepatocyte $[Ca^{2+}]_i$ oscillations, and increased frequency has been correlated with increased phosphorylase kinase activity (5). This enzyme phosphorylates glycogen phosphorylase, activating glycogenolysis, and is functionally connected to $[Ca^{2+}]_i$ by interactions with calmodulin. Thus, frequency modulation of hepatocyte $[Ca^{2+}]_i$ oscillations apparently represents a mechanism to control glycogenolysis. NCCE could influence this either directly through Ca^{2+} entry or indirectly through maintenance of intracellular Ca^{2+} stores. It has been proposed that the NCCE pore is a transient receptor potential (TRP) channel (155, 156).

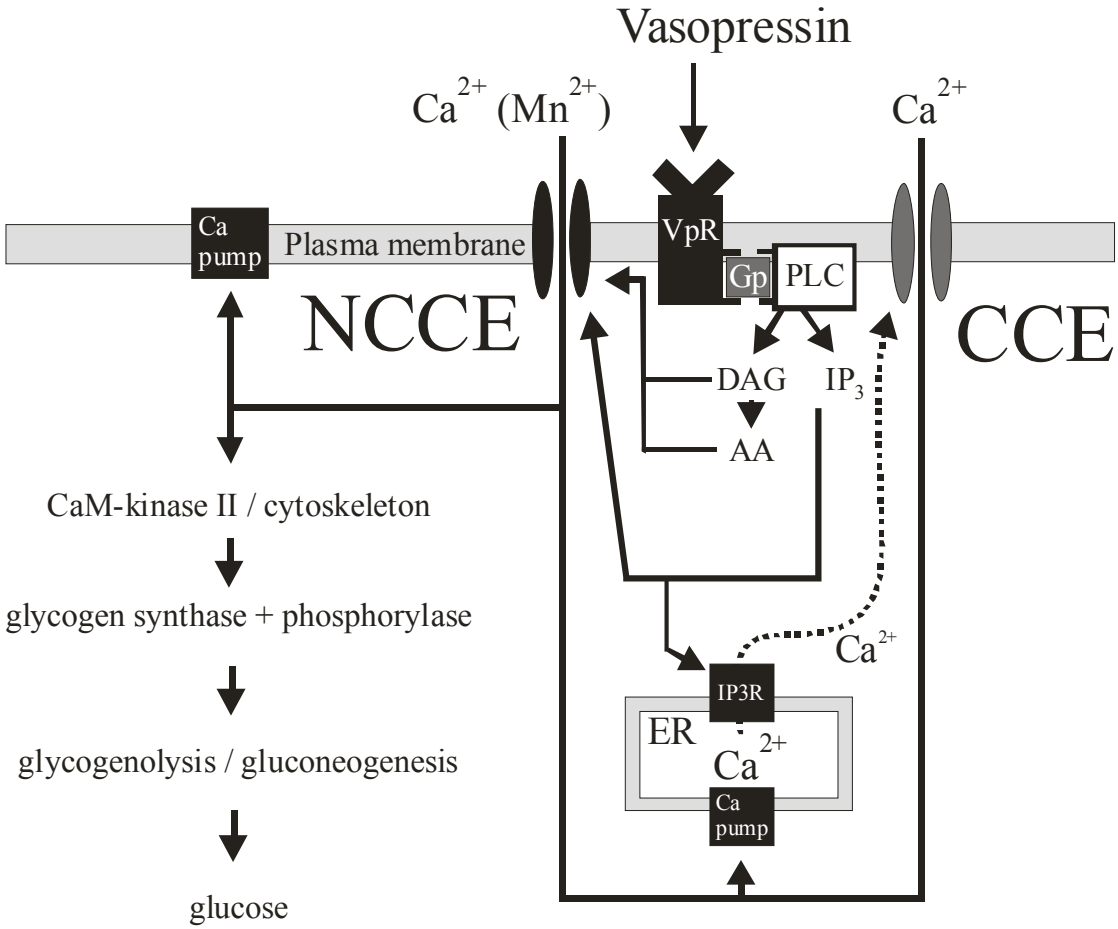

Figure 2. Vasopressin induced noncapacitative Ca^{2+} entry (NCCE) in hepatocytes. Vasopressin binds V1a type vasopressin receptors (VpR) on the hepatocyte plasma membrane, activating intracellular G-proteins (Gp). This triggers (phosphoinositol 4,5-bisphosphate) PIP_2 breakdown to diacylglycerol (DAG) and inositol 1,4,5 trisphosphate (IP_3) by phospholipase C-β (PLC). Some of the DAG is further converted to arachidonic acid (AA). DAG, IP_3, and AA have been shown to activate NCCE. For comparison, the capacitative Ca^{2+} entry (CCE) pathway is shown, which is directly activated by elevated $[Ca^{2+}]_i$. The NCCE and CCE pathways co-exist within the same cell and are not necessarily mutually exclusive. NCCE could directly be involved in $[Ca^{2+}]_i$ oscillations or in refilling and maintaining endoplasmic reticulin (ER) Ca^{2+} stores. Whereas hepatocyte CCE is highly selective for Ca^{2+}, the NCCE pathway is permeant to both Ca^{2+} and Mn^{2+}. Putative mechanisms by which vasopressin triggers glycogenolysis and gluconeogenesis are shown. It is generally accepted that in hepatocytes: 1) vasopressin acts via PLC linked V1a receptors, 2) unlike glucagon, vasopressin does not increase cAMP, and 3) potently stimulates glucose output.

Discussion of Paper II

One novel finding of Paper II was that microinjection of NAD into normal murine pancreatic β-cells does not yield an increase in $[Ca^{2+}]_i$ similar to what was described in sea urchin eggs (144). None of three classical stimuli of β-cell $[Ca^{2+}]_i$ transients (CCh acting on ER Ca^{2+} stores, KCl acting on voltage gated Ca^{2+} channels or glucose acting through metabolism) were affected by either competitive inhibition of cADPR action by $8NH_2$-cADPR or desensitization

by prior cADPR treatment. The suppliers confirmed that both cADPR and $8NH_2$-cADPR were active, and pH of 7.0 was double-checked with pH paper prior to microinjection. These results, taken together with the earlier publication from this lab (91) showing no Ca^{2+} mobilizing action of cADPR in permeabilized *ob/ob* islets, rejected a direct role for cADPR in the mechanisms linking physiological glucose changes to insulin secretion.

The findings of Paper II should not be taken to mean that cADPR does not exist or change concentration in the pancreatic β-cell or that it lacks any function. Other roles were not investigated (e.g., effects on gene expression, protein phosphorylation, etc). There are indications that CD38 auto-antibodies in humans may be involved in the pathogenesis of DM by direct action on pancreatic β-cells (157).

Nicotinamide is a potent inhibitor of cADPR synthesis and action (158-161). Caged cADPR has been shown to increase $[Ca^{2+}]_i$ from Thaps sensitive stores in MIN6 insulinoma cells (162), but no data were presented to show whether blocking this, as by nicotinamide, influences insulin secretion. Nicotinamide mildly potentiates glucose induced insulin secretion in MIN6 cells rather than inhibiting it, while no effect has been detected in *ob/ob* preparations (unpublished observation). No influence of nicotinamide in cultured *ob/ob* islets has been found at concentrations now known to inhibit cADPR (163). The different nicotinamide responses of MIN6 and *ob/ob* β-cells might someday prove helpful in understanding why some cells, but not others, respond to cADPR.

Takasawa *et al* (164) did not find any potent Ca^{2+} mobilizing action of cADPR in islet microsomes as compared to IP_3 induced mobilization from 3 months old hyperglycemic *ob/ob* mice, consistent with the conclusions of Paper II using the local *ob/ob* mice at 10-12 months of age. Glycemic status, which would be expected to differ at 3 and 10 months of age, does not appear to correlate with absence of cADPR action in *ob/ob* mice.

Another messenger synthesized by CD38, nicotinic acid adenine dinucleotide (NAADP, ref. 165), has been seen to evoke changes in $[Ca^{2+}]_i$ in human pancreatic β-cells (166). This is an important observation. It is known that when CD38 is actively synthesizing NAADP, this involves an acidic environment in which cADPR synthesis is potently inhibited (165,167) and further suggests subcellular localization of CD38.

It is widely believed that cADPR is an endogenous ligand of the RyR. There are three known isoforms (RyR1, Ryr2 and RyR3, reviewed in ref. 168), and these are the products of three different genes. Using RNase protection assay, Islam *et al* (169) only identified transcription of RyR2 in *ob/ob* mouse islets and βTC3 insulinoma cells. The transcription level in *ob/ob* islets was ~1000 times lower than in heart. Different investigators have obtained different results. Using PCR of cDNA, Mitchell *et al* (170) found both RyR1 and RyR2 in MIN6, INS-1 cell lines and rat islets. The expression level of RyR1 was only ~5 times lower than in heart. Similar results were obtained with Western blot of MIN6 cell extracts. Using antibodies selective for the three RyR's, Gamberucci *et al*[1] (171) found mainly RyR2 in INS-1 and essentially no RyR's of any kind in RINmF5 rat insulinoma cells that lack glucose induced insulin secretion. Holz *et al* (172) obtained RT-PCR products for RyR2 in rat islets and obtained significant products from 2 of 4 primer sets against RyR1 in βTC3 insulinoma cells, but did not obtain such RT-PCR products from rat islets. Sequencing confirmed the signals

[1] Of interest to Paper I, the Gamberucci *et al* study found none of the three ryanodine receptors in rabbit hepatocyte extracts. These extracts were in fact used as negative controls.

detected in βTC3 cells with the RyR1 primers to be genuine RyR1. Given this assortment of findings, it seems premature to correlate absence of $[Ca^{2+}]_i$ mobilizing effects of cADPR in *ob/ob* β-cells with absence of any particular RyR subtype (i.e., RyR1). Another challenge to the proposed role of cADPR in glucose induced insulin secretion is that rat islet cADPR content is not glucose dependent (173). It should be noted that IP_3 receptors have long been known to mediate CICR (174), and that CICR involving ryanodine sensitive ER Ca^{2+} stores in *ob/ob* islets has also been demonstrated (175). The absence of Ca^{2+} release by cADPR in the *ob/ob* β-cell is therefore not synonymous with absence of CICR.

Formal evidence of functional RyRs (as by ryanodine itself) in human islets was only recently published (166). It is noteworthy that the basic conclusions of that study and an earlier study (176) explicitly support the findings of Paper II. Johnson *et al* (166) confirmed that the CD38/cADPR/RyR pathway is not directly involved in glucose induced insulin secretion. However, ryanodine stimulated insulin secretion by two different mechanisms that were both distinct from that stimulated by glucose. Both NAADP pathways appeared to be non-paracrine and glucose independent.

Discussion of Paper III

DHA was applied to dispersed *ob/ob* cells in order to determine if entry into metabolism beyond the PFK step could trigger $[Ca^{2+}]_i$ oscillations, and if so, if glucose flux was also required. As shown in Paper III figure 1, it was clear that DHA, in the absence of glucose, could in some cases generate an initial $[Ca^{2+}]_i$ response, but this was not followed by oscillations. As shown in Paper III figure 2, in the presence of a substimulatory glucose concentration (4 mM) DHA could generate slow $[Ca^{2+}]_i$ oscillations. The question arose as to whether double the concentration of DHA, so as to compensate for having half as many carbons as glucose, could elicit oscillations. Even at 20 mM DHA, the substimulatory 4 mM glucose was still required (Paper III, figure 3). Collectively, these data were consistent with the model in which PFK-M drives oscillations.

The concomitant measurements of ATP/ADP ratio and slow $[Ca^{2+}]_i$ oscillations shown in Paper III figure 4 clearly demonstrated a very tight temporal coupling between these two parameters. Note that the swings in both parameters are in phase, consistent with the PFK-M model in which oscillating ATP/ADP ratio dictates slow $[Ca^{2+}]_i$ oscillations.

Paper III did not specify how DHA enters the β-cell or what limits its metabolism. It is known that islet glycolytic flux is not limited by glucose transport (177), but for DHA this is not known. Regardless of the entry mechanism, the low triokinase activity described in islets (125) might in any case be rate limiting. Also, [F16BP] varied both with glucose and DHA, sometimes increasing and other times decreasing. It was not possible to obtain sufficient data points to identify oscillations in [F16BP]. There is admittedly some uncertainty regarding the exact mechanism of action and fate of DHA. Radio-labeled DHA would be useful in this regard, but this was not commercially available as of the time Paper III was submitted.

Discussion of Paper IV

The high degree of preservation of $[Ca^{2+}]_i$ and insulin secretion oscillations in the PFK-M deficient islet preparations, despite the roughly 95% reduction in PFK-M, might be explained by published measurements of stimulated glucose utilization combined with glucokinase and PFK Vmax activity. It has been understood since the early 1960's that a metabolic bottleneck

exists in islets (116). In later studies, the Vmax for PFK activity was found to be 40 times higher than for glucokinase (178). A similar value was seen in a related study, and in addition, glucokinase activity was found to be well above glucose utilization under stimulatory conditions while PFK Vmax was about 100 times higher than stimulated glucose utilization (179). Those findings combined with the PFK-M expression level observed in Paper IV indicate that it is possible for PFK-M deficient mouse islets to have had sufficient residual PFK-M activity to drive oscillations. The impairment seen in mean and amplitude of insulin secretion combined with the essentially complete preservation of $[Ca^{2+}]_i$ oscillations suggested a mild impediment in metabolic flux that is more deleterious to the machinery of exocytosis than to that of $[Ca^{2+}]_i$ oscillations. Measurements of ATP/ADP ratio could not detect this.

In view of the data from the PFK-M deficient mice, it would be interesting to examine and compare some aspects of liver glycolysis and also the effects of altered relative amounts of PFK-L in pancreatic β-cells. It has long been known that while the major PFK isoform in liver is PFK-L, there is an additional component (180) that is now known to be PFK-M (181). This brings to mind two questions regarding modulation of glycolytic flux: 1) does the hepatocyte possess intrinsic metabolic oscillations driven by the minority population of PFK-M subunits, and if not, 2) does PFK-L over-expression in pancreatic β-cells dampen oscillations in that cell type?

The results obtained for the PFK-M deficient mice differ somewhat from what was reported for humans with global loss of functional PFK-M (type VII glycogenosis or Tarui's disease, 182). One major difference is that homozygous humans lack wildtype PFK-M altogether, while the PFK-M deficient mice had a tissue specific reduction of enzymatically functional wildtype PFK-M. Mutations and truncations in PFK-M can lead to loss of activity, loss of allosteric regulation and alterations in protein-protein interactions affecting either PFK-M or perhaps the function of the other isoforms through derangements in cooperativity. The impairment in oscillations seen in the heterozygous human siblings could be the result of disruptive activity of tetramers caused by incorporation of truncated mutant PFK-M protein. Thus, the PFK-M deficient mice described in Paper IV are not comparable to the human disorder.

It is possible that futile cycling at the PFK step of glycolysis may have been enhanced as a result of a reduced PFK/F16BPase ratio. While PFK-M was reduced in the β-cells, the reverse activity conducted by F16BPase could have remained unchanged. There are no reports of futile cycling at the PFK step in the β-cell, and limited data elsewhere, but cycling at this glycolytic step has always been found when it was assayed (183-185).

No direct connection between changes in $[Ca^{2+}]_i$ and activity of metabolic enzymes was discussed in Papers III or IV. It was shown many years ago that glycolysis can be acutely stimulated by Ca^{2+} (117-119), while purified PFK-M was shown not to be directly activated by this cation (119). In terms of cation effects on PFK activity, the enzymatic characterization of brain PFK by Muntz (186) is perhaps the most thorough and the most relevant to islet PFK, given the similar levels of all three isoforms in both tissues. While Ca^{2+} was not studied, Muntz found that all cations studied affected brain PFK activity with K^+ (in crude extracts), Mg^{2+}, Mn^{2+}, Co^{2+} and NH_4^{2+} being activators and Cu^{2+} and Ag^+ being potent inhibitors. Zn^{2+} slightly activated at low concentrations and inhibited at higher concentrations. It was later shown that calmodulin binds two sites on PFK-M with high affinity (K_D 11 and 198 nM) and inhibits this enzyme (112). This interaction affected dimerization/tetramerization interconversion and activity levels that were Ca^{2+} dependent, with elevated $[Ca^{2+}]$, MgATP and F16BP giving re-activation following prior inhibition by calmodulin. For comparison, models have been put

forward depicting hepatocyte regulation of glycolysis and gluconeogenesis in which increased $[Ca^{2+}]_i$ inhibits glycolysis at the PFK step by cytoskeletal disassembly (187). Similar to islet extracts, the bulk of PFK-M in muscle extracts also appears to be inactive because PFK-M Vmax is hundreds of times above maximum glucose utilization under stimulatory conditions (187). The large excess of functional PFK-M expression above glucose utilization seems to be a common phenomenon. Cyclic activation and inactivation of PFK-M by oscillating $[Ca^{2+}]_i$ acting on calmodulin or the cytoskeleton potentially represent a means of re-enforcing metabolic oscillations and therefore could someday be incorporated into the PFK-M model. There remain many basic questions about PFK-M regulation, especially in the intact cell where many cations coexist in a dynamic environment.

Paper IV did not examine *in vivo* insulin pulsatility. To do so in a mouse would require significant time and resources, as evidenced by the scarcity of publications in which this was done. The first description of *in vivo* insulin oscillations in the mouse was published only at the final stages of writing this thesis (189). The pattern of the *in vivo* insulin oscillations were mimicked by $[Ca^{2+}]_i$ oscillations *in vitro*, demonstrating the tight temporal connection that normally exists between $[Ca^{2+}]_i$ and insulin oscillations.

Discussion of F26BP study

The fact that no dampening of glucose induced $[Ca^{2+}]_i$ oscillations following microinjection of F26BP was observed could be explained by different means. It is clear that the injected Fura-2 with its carboxyl moieties not only rapidly diffused throughout the cell, but accumulated in the nucleus within no more than a few seconds. There is no way to know with certainty if F26BP, also negatively charged, shared this fate. Rapid degradation of F26BP by a non-specific phosphatase activity has never been described in any cell type and islet fructose 2,6-bisphosphatase (F26BPase) activity is thought to be very low (190). F26BP is highly sensitive to acid hydrolysis, but as indicated earlier, the pH of the β-cell cytosol, where the injected F26BP is expected to localize and have its effect on PFK-M, tends to be roughly neutral and rises transiently with glucose stimulation (Paper III, figure 8A).

The F26BP microinjection results highlight a cautionary note in interpreting published data of glucose induced increases in [F26BP] in insulin secreting cells to mean that the PFK2/F26BP pathway regulates PFK-M activity. One mechanism by which increased [F26BP] would fail to activate or stabilize PFK-M activity under conditions in which F16BP could still evoke its allosteric action would be in the presence of citrate. The affinity of F26BP for the allosteric site of PFK-M is more sensitive to [citrate] than is F16BP affinity (191). A couple of reports have described [citrate] in the islet (192,193). Citrate was found to accumulate in *ob/ob* islets, reaching levels twice as high as lean mice (193). Hence, it seems possible for PFK-M to drive metabolic oscillations with limited susceptibility to changing F26BP levels. It was therefore concluded that, assuming PFK-M is the intrinsic oscillator, in the pancreatic β-cell allosteric regulation of PFK-M activity at the autocatalytic site occurs solely by F16BP, as opposed to co-regulation together with F26BP. The two highest microinjection concentrations tested here are well in line with the highest F26BP concentrations reported by Sener *et al*. (129), yet $[Ca^{2+}]_i$ oscillations were not dampened.

Sener *et al* (129) found that islet [F26BP] increases with glucose stimulation, while Burch *et al* (130) did not. The reason for this discrepancy is not clear. Perhaps if the glucose concentration is maintained at a very low level prior to glucose stimulation, as in the Sener *et al* report, there is an initial overshoot in [F26BP] in response to high glucose, which over time would fall to a

stable equilibrium. In the present experiments, where glucose remained at 11 mM for roughly 48 hours, [F26BP] never exceeded 3 μM, in line with the findings of Burch *et al*. The 45 minutes incubation, when loading Fura-2 AM at 3 mM glucose, would not be expected to be sufficient to lower F26BP levels because of the low F26BPase activity thought to exist in islets (190).

CONCLUSIONS

Reflecting back at the aims of this study, the following conclusions were drawn:

Paper I The hepatocyte response to vasopressin involves at least one Ca^{2+} entry pathway in addition to the capacitative pathway. This non-capacitative Ca^{2+} entry pathway differs from the capacitative one in that it is not activated merely by a rise in $[Ca^{2+}]_i$ but requires activation of a G-protein coupled receptor and is permeable to Mn^{2+} as well as Ca^{2+}.

Paper II A direct role for cADPR in glucose induced insulin secretion was rejected based on $[Ca^{2+}]_i$ measurements following microinjection of the precursor NAD, cADPR itself or the antagonist $8NH_2$-cADPR as well as assays of insulin secretion in permeabilized β-cells.

Paper III DHA triggers $[Ca^{2+}]_i$ oscillations at substimulatory glucose levels, probably by raising [F16BP] into the range at which PFK-M can oscillate. The obligatory requirement of glucose in order to get these oscillations demonstrated the importance of fuel carbons to enter metabolism through the first three steps of glycolysis.

Paper IV Disruption of the distal promoter of the *pfkm* gene leads to tissue specific loss of PFK-M activity and glucose intolerance. The loss of 95-98% of PFK-M activity in islets was correlated with impaired insulin secretion without detectable impairment in glucose induced $[Ca^{2+}]_i$ oscillations or ATP/ADP. If glycolytic derangements exist in these islets, they are probably very subtle. Just as with skeletal muscle, the pancreatic islet harbors a vast excess of functional PFK-M.

Unpublished (F26BP in the pancreatic β-cell) Increasing [F26BP] by microinjection does not dampen $[Ca^{2+}]_i$ oscillations. Under the conditions used for these experiments, with cell culture at 11 mM glucose, endogenous [F26BP] was at or below 3 μM. In the pancreatic β-cell, the autocatalytic site of PFK-M is more likely regulated by F16BP alone as opposed to being co-regulated by F16BP and F26BP. This may be explained in part by the high citrate levels in this cell type that would reduce affinity of F26BP for the autocatalytic site combined with limited synthesis of F26BP.

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