HORMONAL REGULATION OF HEPATIC GLUTAMINASE

STEPHEN ARTHUR SQUIRES

Hormonal Regulation of Hepatic Glutaminase

by

c Stephen Arthur Squires, B.Sc.

A thesis submitted to the
School of Graduate Studies
in partial fulfillment of the
requirements for the degree of
Master of Science

Department of Biochemistry
Memorial University of Newfoundland

1994

St. John's Newfoundland

ISBN 0-315-96068-X

ABSTRACT

Liver glutaminase, a mitochondrial enzyme, is known to be activated by hormones such as glucagon. Diabetes mellitus is a metabolic disease which is characterized by alterations in the insulin:glucagon ratio and, therefore, represents an *in vivo* situation in which to study the effects of hyperglucagonemia on liver glutaminase. Glutaminase flux was found to be increased in isolated hepatocytes and in isolated mitochondria from streptozotocin-induced diabetic rats.

An important question is how the hormonal signal is transmitted into the mitochondria. One possibility examined is whether cAMP-mediated phosphorylation of cytosolic proteins can stimulate a mitochondrial enzyme. This was examined using the cell-permeable protein phosphatase inhibitors okadaic acid and calyculin A. In the presence of 1 mM NH_4Cl, an obligatory positive effector for liver glutaminase, both inhibitors increased glutaminase flux in isolated hepatocytes. This effect was stable, existing in mitochondria isolated from okadaic acid-treated hepatocytes. The protein kinase A agonist, Sp-cAMPS, was also found to stimulate glutaminase flux in isolated hepatocytes. This stimulation was inhibited by the protein kinase A antagonist, Rp-cAMPS. These results suggest that the cAMP-dependent phosphorylation of cytosolic protein(s) can affect mitochondrial glutamine metabolism.

This may also give insight into the mechanisms whereby hormones, such as glucagon, stimulate mitochondrial glutamine metabolism.

ACKNOWLEDGEMENTS

I wish to acknowledge the support and patience of my supervisor, Dr. J.T. Brosnan. Special thanks is also given to my supervisory committee of Dr. M.E. Brosnan and Dr. M. Jois.

I wish to thank the following people for their support, guidance, and helpfulness during the course of my research: Beatrice Hall, H. Stephen Ewart, Dalong Qian, Srinivas Dhanakoti, Stephen Day, Susan Skanes, Doug Bartlett, and Tina Wells. I would also like to thank all of the members of the Biochemistry Department at Memorial for any and all assistance given.

No research can be completed without funding. In this regard, I acknowledge the Canadian Diabetes Association, the Medical Research Council of Canada, and the School of Graduate Studies at Memorial University.

In conclusion, I wish to dedicate this thesis to my parents. Thank you both very much for your support, understanding, and guidance over the past three years. Without your extra push this thesis may never have been completed.

TABLE OF CONTENTS

List of Figures

List of Tables

LIST OF ABBREVIATIONS

ADP ----- adenosine diphosphate
ATP ----- adenosine triphosphate
cAMP ---- cyclic adenosine-3',5'-monophosphate
cAMPS --- cyclic adenosine-3',5'-monophosphothiorate
BSA ----- bovine serum albumin
DMSO ---- dimethyl sulfoxide
EDTA ---- ethylenediaminetetraacetic acid
EGTA ---- ethylene glycol-bis(ß-aminoethyl ether) N,N,N',N'-tetraacetic acid
Hepes --- N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]
MSO ----- methionine sulfoximine
NAD^+----- nicotinamide adenine dinucleotide (oxidized form)
NADH ---- nicotinamide adenine dinucleotide (reduced form)
TH-glucagon - 1-N-α-trinitrophenylhistidine, 12-homoarginine glucagon

Chapter 1

Introduction

INTRODUCTION

Glutamine is a nonessential amino acid with several functions in mammalian systems. It is the most abundant amino acid in blood at a concentration of 0.5 to 0.6 mM (Brosnan et al., 1983). Glutamine serves in the biosynthesis of purines, pyrimidines, glucosamine, and NAD. In liver it is a precursor for glucose and for urea; in kidney it is a precursor for glucose and ammonia. Glutamine synthesis serves to protect the brain against ammonia toxicity and glutamine can act as a precursor for certain neurotransmitters. Cells such as enterocytes, lymphocytes, reticulocytes, oocytes, and cancer cells use glutamine as a respiratory fuel.

GLUTAMINASE

History

In 1935 Krebs first discovered that extracts of mammalian brain, liver, and kidney could cause deamidation of glutamine (Krebs, 1935). Using differences in kinetics, he also identified two different glutaminase activities (liver- and kidney-type). In the 1940's, further studies by Errera and Greenstein showed that the activity called glutaminase I was activated by inorganic phosphate and that this enzyme was located within the mitochondria (Errera and Greenstein, 1949). Subsequently, Guha (1962) was able to show that liver glutaminase was exclusively found in mitochondria. Further

studies were conducted to determine the location of glutaminase within the mitochondria. Using sonication or the addition of detergents, Kalra and Brosnan (1973) found that glutaminase was released from liver mitochondria to the same extent as matrix marker enzymes. From this they concluded that liver glutaminase was located in the mitochondrial matrix. McGivan and co-workers used liver mitochondria disrupted by freezing and thawing and found that glutaminase was associated with the mitochondrial membrane fraction (McGivan et al., 1980). Using intact pig renal mitochondria, Kvamme et al. (1991) suggested that glutaminase has a predominantly external localization on the inner mitochondrial membrane. Their conclusion was taken from experiments in which sulfhydryl, (SH), reagents which may not penetrate the inner mitochondrial membrane still caused an inhibition of glutaminase. However, this location is not generally accepted since there are difficulties with the permeablity of SH reagents and their reactivity with transporters involved in glutamine metabolism.

General properties

Glutaminase (L-glutamine amidohydrolase) (EC 3.5.1.2) is also known as glutaminase I, phosphate-activated glutaminase, and phosphate-dependent glutaminase. It catalyses the following irreversible reaction:

$$\text{L-glutamine} + H_2O \rightarrow \text{L-glutamate}^- + NH_4^+$$

The activity originally described as glutaminase II is actually a combination of glutamine-oxo-acid amidotransferase and ω-amidase. It consists of the following two reactions:

$$\text{glutamine} + \alpha\text{-ketoglutarate}^{2-} \rightleftharpoons \text{2-oxoglutaramate}^- + \text{glutamate}$$

and

$$\text{2-oxoglutaramate}^- + H_2O \rightarrow \alpha\text{-ketoglutarate}^{2-} + NH_4^+$$

with a net reaction of:

$$\text{L-glutamine} + H_2O \rightarrow \text{L-glutamate} + NH_4^+$$

Glutaminase will be used in this thesis to represent the EC 3.5.1.2 enzyme. There are, however, two isoenzymes of glutaminase. This was first demonstrated by Krebs in 1935 (Krebs, 1935).

Kidney-type glutaminase

Kidney-type glutaminase is found in kidney, brain, intestine, skeletal muscle, heart and lung. It is also found in fetal liver, tumours, lymphocytes, and reticulocytes. This isoenzyme of glutaminase is activated by phosphate (Errera and

Greenstein, 1949) and is inhibited by glutamate. The inhibition by glutamate is noncompetitive with glutamine but is competitive with phosphate (Sayre and Roberts, 1958). The Km for glutamine is 4 to 5 mM for the kidney enzyme (Klingman and Handler, 1958), 2 to 8 mM for the brain enzyme (Svenneby et al., 1973) and 2.2 mM for the intestinal enzyme (Pinkus and Windmueller, 1977).

Other compounds can also affect kidney-type glutaminase. This glutaminase can be activated by ATP, ADP, ITP, CTP, and some carboxylic acids such as succinate, α-ketoglutarate and malate (see Kovacevic and McGivan, 1983). Sulfhydryl (SH)-blocking reagents such as mercuric chloride, p-chloromercuribenzoate (PCMB), mersalyl, N-ethylmaleimide, and 3,3'-dithiobis[6-nitro-]benzoic acid (DTNB) inhibit the kidney-type enzyme (Kvamme et al., 1970).

In the kidney, glutaminase activity increases during metabolic acidosis (Curthoys et al., 1976) and streptozotocin-induced diabetes (Watford et al., 1984). It is also increased by feeding diets high in protein (Brosnan et al., 1978). If the acidosis associated with these situations is corrected by $NaHCO_3$ administration, the glutaminase activity does not increase (Brosnan et al., 1978; Watford et al., 1984).

Liver type-glutaminase

Liver-type glutaminase is found only in adult liver. It is not inhibited by glutamate (Krebs, 1935) as is the case for kidney-type glutaminase. However, like the kidney-type glutaminase, the liver-type is inhibited by SH-blocking agents (Joseph and Meijer, 1981). Smith and Watford (1988, 1990) have been able to purify and characterize rat liver-type glutaminase. They report an apparent subunit molecular mass of 58,000 Da with a Km for glutamine of 17 mM and a pH optimum of 7.8 to 8.2 (Smith and Watford, 1988).

Intercellular glutamine cycling and ammonia detoxification

The functional units of the liver are the so-called acini. An acinus represents a cluster of parenchymal cells which are grouped about terminal branches of the hepatic arteriole and portal venule (Lautt and Greenway, 1987). The vascular stalk enters the center of the acinus where the hepatic arterial and portal venous blood are well mixed within the periportal zone. All entrances to the acinus occur in the periportal region, while all exits occur at the periphery. The acini extend from the terminal portal venule to the terminal hepatic venule. The cells near the portal venule are referred to as the periportal hepatocytes. These periportal hepatocytes are supplied with blood that is rich in oxygen, hormones and substrates. The smaller group of cells, known as perivenous hepatocytes, are located near the hepatic venule.

This perivenous zone is supplied by blood which is poor in oxygen but rich in carbon dioxide and metabolic products.

Immunohistochemical studies indicate that carbamoyl-phosphate synthetase I (Gaasbeck-Janzen et al., 1984) and glutamine synthetase (Gebhardt and Mecke, 1983) are localized exclusively in the periportal and perivenous area of the liver lobule, respectively. Using the perfused rat liver, Häussinger has been able to demonstrate the importance of hepatocyte heterogeneity in glutamine and ammonia metabolism (Häussinger, 1983). This work has suggested a role of an intercellular glutamine cycle during ureagenesis in perfused rat liver.

Häussinger's studies involved perfusing rat liver in the physiological antegrade (portal to caval vein) and in retrograde (caval to portal vein). He found that added ammonium ions at low concentrations are mainly converted to urea in antegrade perfusion and to glutamine in retrograde perfusions (Häussinger, 1983). These studies support the concept of cellular heterogeneity in liver. Further evidence of this is provided by the observation that when perivenous hepatocytes are selectively destroyed by a dose of CCl_4, the livers fail to synthesize glutamine and to take up glutamate (Häussinger et al., 1983; Gebhardt et al., 1988).

The organization of the liver cells and of the enzymes involved in ammonia detoxification is physiologically

important. The portal blood first comes into contact with hepatocytes capable of urea synthesis before glutamine-synthesizing cells in the perivenous zone are reached. The rate-limiting enzyme of the urea cycle is carbamoyl-phosphate synthetase. It has an higher Km for total ammonia (NH_3 and NH_4^+) than does glutamine synthetase (Cohen et al., 1985). Therefore, it may be said that the periportal zone represents a low-affinity, high capacity system for ammonia detoxification compared to the high-affinity perivenous zone (Häussinger, 1990). Ammonia at a physiological portal concentration of 0.2 to 0.3 mM is converted by about two-thirds into urea and one-third into glutamine (Häussinger, 1990).

Hepatic Transport of Glutamine

Glutamine is transported into hepatocytes by a Na^+-dependent transport system. Kilberg et al.(1980) first described this system and named it system N. In addition to glutamine, this system also transports asparagine and histidine. A 100-kDa plasma membrane protein may mediate system N transport in rat hepatocytes (Tamarappoo et al., 1992). Glutamine can also be transported via system A, although this transport is estimated to represent only 5 to 10 % of total hepatic glutamine transport (Low et al., 1991). Na^+-independent transport of glutamine is involved in efflux

of glutamine from perivenous hepatocytes (Fafournoux et al., 1983; Burger et al., 1989).

System N transport of glutamine is not affected by glucagon or insulin in short term hepatocyte incubations (Kilberg et al., 1980) but induction does occur with prolonged exposure of cell cultures to hormones (Gebhardt and Kleeman, 1987). Corticosteroid treatment of rats was found to increase system N activity in liver sinusoidal membrane vesicles (Low et al., 1992). Streptozotocin-induced diabetes causes an increase in system N activity in isolated hepatocytes (Barber et al., 1982) but this was not found in sinusoidal membrane vesicles (Low et al., 1992). Decreasing the extracellular pH also causes a decrease in glutamine uptake in perfused rat liver (Lenzen et al., 1987).

System N activity is high in liver. This is indicated by the fact that portal blood glutamine is at 0.6 mM while the liver tissue concentration is 4 to 6 mM. Häussinger has found that histidine in near-physiological concentrations inhibits both glutamine uptake by periportal hepatocytes and its release by perivenous hepatocytes (Häussinger et al., 1985). This suggests that glutamine transport can be involved in the regulation of hepatic glutamine metabolism.

Glutamine catabolism occurs in mitochondria mainly through the actions of glutaminase. Glutamine has been shown to equilibrate rapidly across the inner mitochondrial membrane

(Joseph and McGivan, 1978b; Joseph and Meijer, 1981). This is due to a specific carrier which can be inhibited by mersalyl (Joseph and Meijer, 1981). In contrast, Soboll et al. (1991) have also suggested that glutamine uptake into mitochondria is an electroneutral event which is sensitive to changes in pH. This study involved incubating isolated mitochondria with a known concentration of [^{14}C]glutamine and then determining glutamine uptake by measuring both the radioactivity associated with the mitochondrial pellet and the glutamine concentration in the medium. From this they concluded that increasing pH causes an increase in glutamine uptake. Another study by the same group had previously shown that when the pH increases from 7.3 to 7.7 the mitochondrial concentration increases from 15 to 50 mM (Lenzen et al., 1987). These values were obtained by fractionating freeze-clamped liver tissue in non-aqueous solvents and subsequently determining the glutamine concentration in the various fractions. This, in my mind, does not represent an accurate method for determining intracellular glutamine concentrations and may account for the high values obtained.

It is, of course, possible that transport of glutamine across the plasma membrane or across the inner mitochondrial membrane could be rate-limiting in the overall metabolism of glutamine. This has been examined from the point of view of control strength (Pogson et al., 1990; Low et al., 1990).

These studies used L-glutamate-γ-hydrazide to inhibit glutaminase in isolated hepatocytes and in isolated mitochondria. At the concentrations tested, there was no effect of this compound on glutamine transport processes (Low et al., 1990). The degree of inhibition of glutamine metabolism in hepatocytes and mitochondria was essentially the same at a given concentration of L-glutamate-γ-hydrazide. The flux control coefficent exerted by glutaminase was, therefore, equal to 1.0 (Pogson et al., 1990) suggesting that transport processes were not rate limiting. Repeating these experiments using isolated hepatocytes in the presence of histidine showed that the flux control coefficent of glutamine uptake by system N was 0.31, and therefore as previously stated by Häussinger et al. (1985), transport across the plasma membrane could exert a regulatory influence on glutamine metabolism (Pogson et al., 1990).

REGULATION OF LIVER-TYPE GLUTAMINASE

Activators of liver-type glutaminase

Liver glutaminase has an interesting property in being activated by one of its products, namely ammonia. This was first reported for intact liver mitochondria (Charles, 1968) and has subsequently been observed in isolated perfused rat liver (Häussinger et al., 1983) and hepatocytes (Joseph and McGivan, 1978a). Ammonia stimulation of glutaminase is found

in mitochondria that have been disrupted by freezing and thawing (Joseph and McGivan, 1978b; Verhoeven et al., 1983; Szweda and Atkinson, 1989). This indicates that ammonia acts directly on the enzyme.

The exact role of bicarbonate in regulating liver glutaminase is still uncertain. Contradictory reports have bicarbonate either activating or having no effect on liver glutaminase. In isolated mitochondria (Joseph and McGivan, 1978b), and in isolated hepatocytes (Baverel and Lund, 1979) bicarbonate is reported to act as an activator of glutaminase. In mitochondria disrupted by freezing and thawing, HCO_3^- activated glutaminase by decreasing the half-maximal phosphate requirement without affecting the maximal velocity of the enzyme (McGivan et al., 1980). In the absence of HCO_3^- or NH_4Cl, glutaminase assayed in freeze-thawed mitochondria required 25 mM Pi for half-maximal activity while in the presence of HCO_3^- or NH_4Cl this Pi requirement was reduced to 4mM (McGivan et al., 1980). Using a semi-purified glutaminase preparation, Patel and McGivan (1984) were not able to show a stimulation by HCO_3^-. However in the isolated perfused rat liver, glutaminase was not affected by the presence of HCO_3^- in the perfusate (Häussinger et al., 1980). It can therefore

be said that the role of bicarbonate in regulating liver glutaminase is still uncertain.

Several other substances have been found to affect liver-type glutaminase. In isolated hepatocytes, Baverel and Lund (1979) found that leucine at near-physiological concentrations (0.1-1 μM) is an activator of glutaminase. In intact liver mitochondria EDTA stimulates glutaminase and this activation can be reversed by low concentrations of $MgCl_2$ (Joseph et al., 1981). The addition of EDTA affected glutaminase by decreasing the concentration of phosphate required for half-maximal stimulation. They interpreted this to mean that Mg^{2+} actually inhibits glutaminase and its removal by EDTA reverses this inhibition. Using freeze-thawed disrupted mitochondria, glutaminase was not affected by EDTA or $MgCl_2$ (Joseph et al., 1981; McGivan et al., 1985). EDTA and EGTA have been reported to have stimulatory effects on membrane bound glutaminase (Szweda and Atkinson, 1990b). This report also indicates that glutaminase is stimulated by a rise in Mg^{2+} concentration, again due to an increase in the apparent affinity of the enzyme for the positive modifier phosphate (Szweda and Atkinson, 1990b). They also found that increasing the citrate concentration stimulated glutaminase by increasing the

affinity of the enzyme for glutamine (Szweda and Atkinson, 1990b).

In disrupted mitochondria, the NH_4Cl stimulation was increased by the addition of ATP (Joseph and McGivan, 1978b). This was also observed for the HCO_3^- stimulation of glutaminase (Joseph and McGivan, 1978b). In the absence of NH_4Cl and HCO_3^-, ATP caused a lowering of the phosphate concentration required for half-maximal activation of glutaminase (McGivan et al., 1980).

N-acetylglutamate is an essential activator of carbamoyl-phosphate synthetase. It was also shown by Blackburn et al. (1972) that 5 mM N-acetylglutamate stimulates glutaminase in isolated rat liver mitochondria after a lag of 10 minutes. Similar results using intact mitochondria have been claimed by Meijer and Verhoeven (1986). In this study, however, they proposed to manipulate N-acetylglutamate levels only by incubating mitochondria with glutamate and did not actually measure the N-acetylglutamate levels. These investigators were also unable to show an activation by N-acetylglutamate in broken mitochondria. Using purified glutaminase, Smith and Watford (1988) showed that N-acetylglutamate activated glutaminase by about 10%. These results seem to indicate that

N-acetylglutamate has little, or no, affect on liver glutaminase.

In liver, ureagenesis is stimulated by an increase in pH which leads to removal of bicarbonate and its role has recently been seen in preventing alkalosis, as well as removing the toxic ammonia, thus preventing hyperammonemia. Liver glutaminase has been shown to be sensitive to changes in pH, being stimulated by an alkaline pH (Häussinger et al., 1983; Snodgrass and Lund, 1984; Szweda and Atkinson, 1989). The rise in pH stimulates glutaminase by increasing the affinity of the enzyme for glutamine with no effect on the maximal rate (Snodgrass and Lund, 1984; Szweda and Atkinson, 1989). The increase in glutaminase activity due to increasing pH may also be mediated by increases in the affinity of the enzyme for its positive modifiers, ammonia and phosphate (Szweda and Atkinson, 1989). This is shown by the observation that the concentration of NH_4^+ required for half-maximal rates decreases as pH increases (Szweda and Atkinson, 1990a).

Hormonal regulation of liver-type glutaminase

Liver glutaminase has been demonstrated to be regulated by hormones. In isolated hepatocytes, Joseph and McGivan

(1978a) showed that glucagon stimulated gluconeogenesis from glutamine to a far greater extent than from any other amino acid precursor. They also found that glucagon caused a decrease in intracellular glutamine and an increase in glutamate. This was attributed to an activation of glutaminase by glucagon. Verhoeven et al. (1985) found an α-adrenergic stimulation of glutamine metabolism in isolated rat hepatocytes. Later, Vincent et al. (1989) were also able to demonstrate an increase in glutaminase flux in isolated hepatocytes due to added vasopressin. Using perfused rat liver, Häussinger et al. (1983) found that glutaminase flux was increased by ammonium ions or glucagon with the effects being additive.

Liver mitochondria isolated from rats injected with glucagon have been shown to have altered metabolism compared to control mitochondria from saline-injected rats. Mitochondria from glucagon-injected rats have higher rates of pyruvate carboxylation and decarboxylation (Adam and Haynes, 1969; Garrison and Haynes, 1975), citrulline synthesis (Yamazaki and Graetz, 1977; Hengsens et al., 1980), and glycine oxidation (Jois et al., 1989) compared to mitochondria from saline-injected controls. Lacey et al. (1981) also found that injecting rats with glucagon caused an increased

effective activity of glutaminase in subsequently isolated liver mitochondria. This glucagon effect was seen to decrease the requirement of glutaminase for phosphate. The effect was also lost on disruption of the mitochondria which suggests that the integrity of the mitochondrial membrane is important for the glucagon effect (Lacey et al., 1981).

Hormonal effects on mitochondrial metabolism have also been found using mitochondria isolated from hepatocytes previously incubated with hormones. This was first demonstrated by Siess and Wieland (1979) when they showed increased succinate dehydrogenase activity in mitochondria from glucagon-incubated and dibutyryl cAMP-incubated hepatocytes. Covera and Garcia-Sainz (1983) found that by incubating hepatocytes for 10 minutes with hormones and then isolating mitochondria, glutaminase flux was increased in the isolated mitochondria. This was observed for adrenaline, vasopressin, angiotensin II, and glucagon.

In the above studies there were no steps taken to preserve the phosphorylation state of the proteins in the isolated mitochondria. The mitochondria, once isolated, were seen to somehow "retain" the stimulatory effect of the added hormone. This raises the question of exactly how the hormonal signal gets transmitted into the mitochondria and how does it

affect mitochondrial enzymes such as glutaminase. Several ideas have been put forth to answer this question. Halestrap (1989) has cited increases in mitochondrial volumes as playing a role in the hormonal signalling process. Such hormonal increases in volume may be caused by a Ca^{2+}-mediated increase in the electrogenic K^+ entry. Calcium may cause this by increasing the matrix concentration of PPi (pyrophosphate) by inhibiting the matrix pyrophosphatase. The increased PPi then displaces ADP or ATP that is blocking a K^+ channel (Halestrap, 1989). Lacey et al. (1981) also found that incubating mitochondria in hypoosmotic medium produced the same effects as isolating mitochondria from glucagon-injected rats. Other investigators have found that liver mitochondria isolated from glucagon-injected rats do not have increased volume (Wingrove et al., 1984; Jois et al., 1992). The involvement of mitochondrial volume changes in hormonal signalling therefore remains uncertain.

A second proposal suggests that glucagon application stabilizes rather than activates mitochondrial functions. Siess et al. (1981) proposed that mitochondria from glucagon-injected rats are better able to withstand the isolation procedure than are mitochondria from control, saline-injected rats. This view has been opposed by Jensen et al. (1983) who

found that even in crude liver homogenates glucagon treatment caused increases in such processes as carboxylation of pyruvate and citrulline synthesis. Unlike the earlier study, the choice of mitochondrial isolation medium was without effect. Allan et al. (1983) also found that hormonal effects persisted in mitochondria located in permeable filipin-treated hepatocytes. Also similar effects were found in hepatocytes and isolated mitochondria.

Many cytoplasmic events can be affected by phosphorylation processes caused by the action of hormones. There is no direct evidence using ^{32}P-labelled hepatocytes that glucagon causes an increase in the phosphorylation of a mitochondrial membrane protein(s) (Siess and Wieland, 1979; Vargas et al., 1982). This does not necessarily mean that phosphorylation/dephosphorylation of cytosolic protein(s) is not involved in the overall signalling process. It is certainly possible that such cytosolic events can have effects on mitochondrial functions.

DIABETES MELLITUS AND HEPATIC GLUTAMINE CATABOLISM

The term "diabetes mellitus" is used to represent a number of disease states in which hyperglycemia is the most constant metabolic abnormality. From an endocrinological viewpoint, diabetes mellitus is caused by impaired actions of insulin either due to impaired secretion or end-organ unresponsiveness, and to hypersecretion or hyper responsiveness to the actions of counter-regulatory hormones such as glucagon, growth hormone, and catecholamines. Relative hyperglucagonemia is present in all forms of diabetes in which glucose production exceeds glucose disposal and thus results in "endogenous hyperglycemia" (Unger and Orci, 1981). Such glucagon excess causes the hepatic abnormalities of severe diabetes including increased glycogenolysis, increased gluconeogenesis, and increased ketogenesis. This only occurs if the countering actions of insulin are deficient.

Diabetes mellitus results in increased muscle proteolysis which provides amino acid substrates for gluconeogenesis. There is also an increased production of urea by the liver. The involvement of a hyperglucagonemic state in increased urea production has been demonstrated in a recent study (Almdal et al., 1992). In this study, one group of streptozotocin-induced diabetic rats was injected with a specific antibody

against pancreatic glucagon while a second group of diabetics received an injection of nonimmune rabbit serum. Urea synthesis was increased 2.5 fold in the serum treated diabetics while the production in the glucagon antibody treated-diabetics was identical to the non-diabetic controls (Almdal et al., 1992). This lends support for the importance of glucagon in causing changes in hepatic amino acid metabolism in diabetes.

Glutamine is an important glucogenic amino acid in the liver. In the normal physiological state, there is no significant uptake or release of glutamine by the liver (Brosnan et al., 1983). This changes to a net uptake of glutamine in diabetes mellitus (Schrock and Goldstein, 1981; Brosnan et al., 1983). Such an increase in uptake suggests an increased metabolism of glutamine associated with diabetes mellitus.

AGONISTS/ANTAGONISTS USED IN HORMONAL SIGNALLING STUDIES

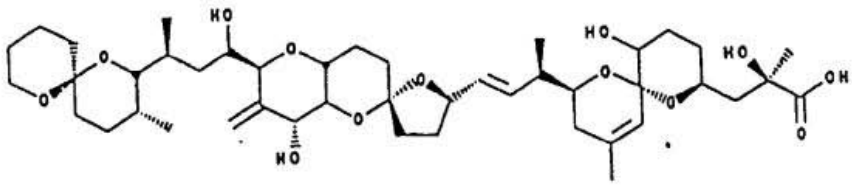
Okadaic acid and Calyculin A

Okadaic acid, ($C_{44}H_{66}O_{13}$), is a complex fatty acid polyketal produced by dinoflagellates (Figure 1.1). It accumulates in the digestive glands of shellfish and marine sponges. One such marine sponge, Halichondria okadaii, was the first to be used in the isolation of okadaic acid and accounts for the origins of its name (Tachibana et al., 1981).

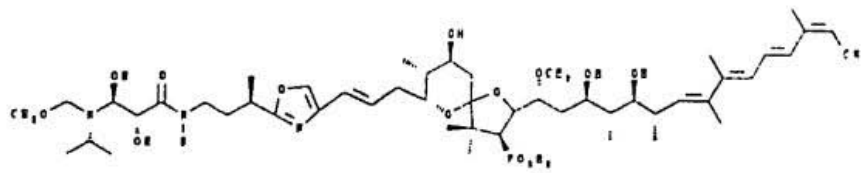
Okadaic acid has been found to cause diarrhetic seafood poisoning. This toxin accumulates in the midgut glands of bivalves feeding on dinoflagellates. Although okadaic acid caused such poisoning, the mechanism of its action was unknown. Takai and co-workers, using isolated vascular smooth muscle, discovered that okadaic acid was a potent inhibitor of protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) (Takai et al., 1987). These are two of the four major protein phosphatases, in the cytosol of mammalian cells, which dephosphorylate serine and threonine residues (Cohen and Cohen, 1989). The actions of okadaic acid are illustrated in Figure 1.2.

Calyculin A contains an octamethyl polyhydroxylated C_{28} fatty acid which is linked to two -amino acids and esterfied by phosphoric acid (Figure 1.1). It was first isolated from

Figure 1.1 Structures of the phosphatase inhibitors okadaic acid and calyculin A



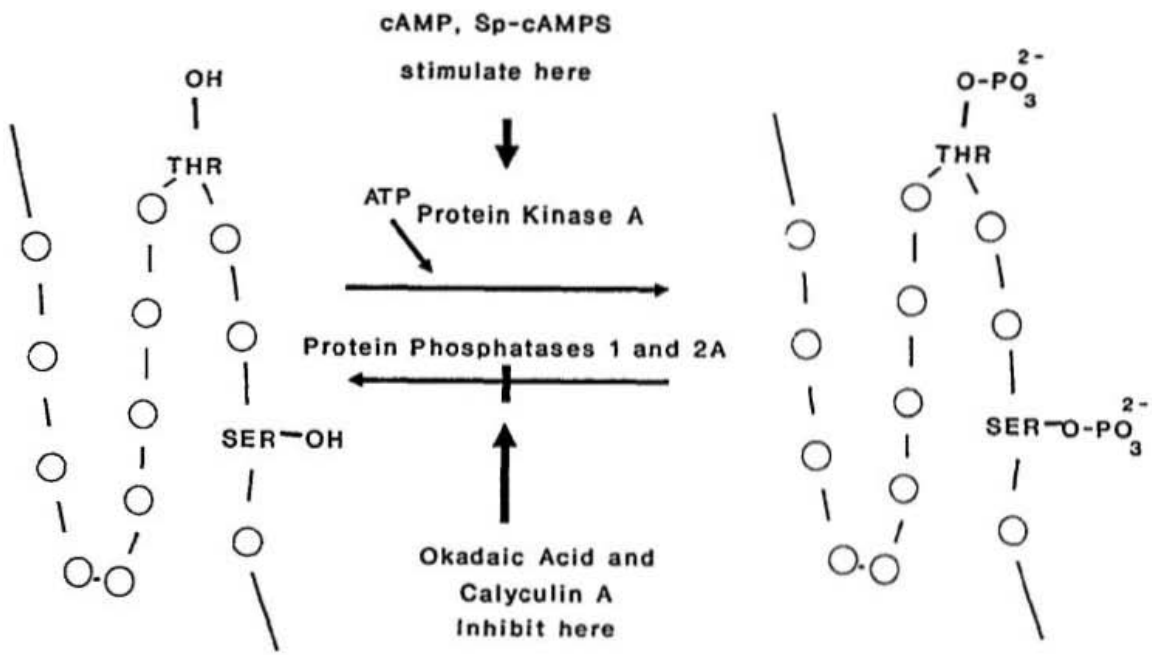
Okadaic Acid



Calyculin A

Figure 1.2. Illustration showing how the phosphatase inhibitors okadaic acid and calyculin A increase the phosphorylation levels of proteins.

Stimuli, such as cAMP, working through protein kinases cause the phosphorylation of threonine and serine residues on proteins. Phosphatases remove the phosphate group from the threonine and serine residues. Okadaic acid and calyculin A increase the level of phosphorylation by inhibiting this removal.


cAMP, Sp-cAMPS
stimulate here
ATP
Protein Kinase A
Protein Phosphatases 1 and 2A
Okadaic Acid and
Calyculin A
Inhibit here
OH
THR
SER—OH
O-PO3 2-
THR
SER—O-PO3 2-

a marine sponge, Discodermia calyx, and is a strong inhibitor of starfish development and has powerful toxic effects on L1210 leukemia cells (Suganuma et al., 1990). Calyculin A, like okadaic acid, inhibits protein phosphatase 1 and 2A (Ishihara et al., 1989) and is cell permeable (Cohen et al., 1990). Its actions are also given in Figure 1.2.

Protein Phosphatase and Okadaic Acid

Cohen and Cohen (1989) classify four major classes of protein phosphatase (PP) catalytic subunits. These are subdivided into type 1 and type 2 with three subtypes of type 2. Type 1 phosphatase, PP1, dephosphorylates the ß-subunit of phosphorylase kinase specifically. It is also inhibited by nanomolar concentrations of the thermostable proteins inhibitor-1 and inhibitor-2. This phosphatase does not have an absolute requirement for divalent cations. Type 2 phosphatases, PP2, dephosphorylate the α-subunit of phosphorylase kinase preferentially and are unaffected by the inhibitors. Protein phosphatase 2A, PP2A, like PP1 does not require divalent cations. Protein phosphatase 2B and 2C are Ca^{2+}/calmodulin- and Mg^{2+}- dependent, respectively. PP2A is completely inhibited by 1 nM okadaic acid, while PP1 requires higher concentrations of 10-15 nM of okadaic acid (Cohen et

al., 1990). The phosphatase inhibitor calyculin A completely inhibits PP2A and PP1 at a concentration of 1 nM (Ishihara et al., 1989).

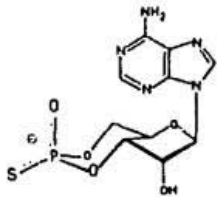
Mitochondrial pyruvate dehydrogenase phosphatase, protein phosphatases that dephosphorylate tyrosine residues, acid and alkaline phosphatases, and inositol trisphosphatase are all insensitive to okadaic acid (Haystead et al., 1989; Cohen et al., 1990). There was also no inhibition of eight different protein kinases tested (Cohen et al., 1990). Using ^{32}P-labelled hepatocytes, the addition of okadaic acid stimulated the overall levels of protein phosphorylation by 2.5 to 3-fold (Haystead et al., 1989; Cohen et al., 1990). Proteins whose phosphorylation increased included rate-limiting enzymes of glycogen and lipid metabolism, glycogenolysis and gluconeogenesis. This included cytosolic enzymes such as acetyl CoA carboxylase, ATP-citrate lyase, pyruvate kinase, and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK2/FBPase) (Haystead et al., 1989). Glycogen phosphorylase and glycogen synthase in the hepatocyte glycogen fraction were also phosphorylated. No increase in protein phosphorylation was observed in the microsomal fraction (Haystead et al., 1989). In this particular study, the mitochondrial fraction was not examined.

In intact cells, namely adipocytes and hepatocytes, higher concentrations of the phosphatase inhibitors are needed than those employed *in vitro*. In most studies the effects of okadaic acid on intact cells were maximal at 1 μM (Haystead *et al.*, 1989; Cohen *et al.*, 1990; Hardie *et al.*, 1991). There are two probable reasons for this. The first is even though okadaic acid and calyculin A are hydrophobic and can penetrate cell membranes, a higher concentration may be needed in the medium to ensure that enough inhibitor gets into the cytosol. A second explanation is that a concentration of 1 μM is necessary because the intracellular concentrations of PP1 and PP2A lie in the range of 0.1-1 μM (Haystead *et al.*, 1989; Hardie *et al.*, 1991).

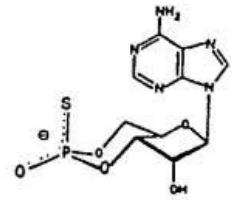
Use of Okadaic Acid in Isolated Cells

Okadaic acid has been used extensively in the study of metabolic regulation in isolated hepatocytes and adipocytes. In hepatocytes, okadaic acid mimicked the action of glucagon by increasing glucose output and conversion of lactate to glucose (Haystead *et al.*, 1989). Okadaic acid also stimulates carnitine palmitoyltransferase I activity and palmitate oxidation in isolated rat hepatocytes (Guzman and Castro, 1991). In isolated adipocytes, okadaic acid stimulated basal

Figure 1.3 Structures of the cAMP analogues Sp-cAMPS and Rp-cAMPS



Rp-cAMPS



Sp-cAMPS

lipolysis and inhibited acetyl CoA carboxylase, the rate-limiting enzyme in fatty acid biosynthesis (Haystead et al., 1989). Okadaic acid also stimulated 2-deoxyglucose uptake by adipocytes (Haystead et al., 1989; Lawerence et al., 1990; Covera et al., 1991). However, okadaic acid inhibited insulin-stimulated glucose transport into adipocytes (Lawerence et al., 1990; Covera et al., 1991). All of these studies indicate that okadaic acid is cell-permeable and phosphorylation / dephosphorylation events play major roles in metabolic regulation. It remains to be established if mitochondrial enzymes such as glutaminase can be affected by cytosolic protein phosphorylation/dephosphorylation events.

cAMP analogues and hormonal signalling

cAMP has poor cell permeability when used with isolated cells but there are a number of analogues of cAMP which have better cell permeability and, depending on their stereochemistry, act as either agonist or antagonist towards protein kinase A. Cyclic adenosine-3',5'-monophosphothiorate, cAMPS, is an analogue of the natural signal molecule cAMP in which one of the two exocyclic oxygen atoms in the cyclic phosphatase moiety is replaced by sulphur. Equatorial thio substitution leads to the R-isomer, while axial modification

yields the S-compound. The structures are given in Figure 1.3. Sp-cAMPS acts as an agonist and Rp-cAMPS as an antagonist towards protein kinase A. The Sp-isomer binds to the holoenzyme of protein kinase A with approximately 10% of the binding affinity of cAMP but fully activates the protein kinase (Rothermel et al., 1983). Binding of the Sp-isomer still allows for the release of the active catalytic subunit from protein kinase A. Rp-cAMPS also binds to the holoenzyme but the Rp-cAMPS-holoenzyme complex does not dissociate to release the free catalytic subunit (Rothermel et al., 1983). Both isomers are very stable against cyclic nucleotide phosphodiesterases (Van Haastert and Kien, 1983).

Sp-cAMPS and Rp-cAMPS have been used in studies involving isolated hepatocytes (Rothermel et al., 1983; Rothermel et al., 1984; Connelly et al., 1987). Sp-cAMPS stimulates glycogenolysis in isolated hepatocytes and this stimulation is inhibited by Rp-cAMPS (Rothermel et al., 1983). Rp-cAMPS has also been used to inhibit glucagon-induced increases in glycogenolysis in isolated hepatocytes (Rothermel et al., 1984; Botelho et al., 1988). This indicates that both cAMPS isomers can be used in probing hormonal signalling events in isolated hepatocytes.

SUMMARY OF TOPICS COVERED

The areas of research covered in this thesis can be divided into two sections. The first section deals with diabetes mellitus and hepatic glutamine catabolism, while the second section covers hormonal regulation and signalling with respect to hepatic glutaminase.

Diabetes represents a physiological situation in which the endogenous circulating glucagon levels are increased and provides a situation where the effects of glucagon on hepatic glutamine catabolism can be studied. Therefore, the possible effects of diabetes mellitus on glutaminase flux in isolated hepatocytes and in isolated liver mitochondria from streptozotocin-induced diabetic rats were studied. If diabetes does result in an increase in glutaminase flux, there is still the question of whether or not insulin treatment of the diabetic rats would prevent or reduce such an increase. This may lend further support for the hormonal regulation of glutaminase. A second question is whether such a stimulation in diabetes is due to more enzyme (Smith and Watford, 1988) or to activation of the enzyme, or to a combination of the two.

As previously stated, hepatic glutaminase is known to be under hormonal control. The major unanswered question is how the hormonal signal gets into the mitochondria and how does it

affect mitochondrial enzymes such as glutaminase. In the second section of this thesis this problem is examined using various cell-permeable agonists and antagonists.

CHAPTER 2
MATERIALS AND METHODS

MATERIALS

Chemicals

Collagenase A was obtained from Boehringer Mannheim (Montreal, Quebec). Okadaic acid and calyculin A were supplied by Biomol Research Laboratories, Plymouth Meeting, PA. Rp- and Sp- cAMPS were purchased from Biolog Life Science Institute, La Jolla, CA. Protamine Zinc insulin (beef and pork PZI) was from Eli Lilly Company (Toronto, Ontario). [1-^{14}C]Glutamate, and Omnifluor were from DuPont New England Nuclear (Mississauga, Ontario). QAE Sephadex A-25 was from Pharmacia LKB, Baie D'Urfe, Quebec. Glucagon, epinephrine and streptozotocin were from Sigma (St. Louis, USA). All other chemicals were obtained from Sigma and were of the highest grade possible.

Preparation of [1-^{14}C] glutamine

To prepare [1-^{14}C]glutamine from [1-^{14}C]glutamate, a 15,000 x g supernatant from a rat liver homogenate of 1 part tissue plus 3 parts 0.9% NaCl was used as a crude preparation of glutamine synthetase. The incubation was for 3 hours at 37° C. The incubation mixture gave a final volume of 0.5 ml and consisted of: Tris/HCl buffer, pH 7.4 (78 mM); NH_4Cl (15.6 mM); $MgCl_2$ (15.6 mM); ATP (7.8 mM); L-[1-^{14}C]glutamate (5.6

mM); phosphocreatine (7.8 mM); creatine kinase (10 units/ml) and crude enzyme (0.2 ml/ml) (Baverel and Lund, 1979). The reaction mixture was deproteinized with 0.05 ml of 10% perchloric acid (w/v). The pH was then adjusted to 7.0 using 50% (w/v) K_2CO_3. [1-^{14}C]Glutamine was separated from any [1-^{14}C]glutamate by column chromatography using Sephadex QAE. This is an anion exchanger which will bind glutamate but not glutamine. This gel had been equilibrated with Tris-HCl (40 mM) pH 5.7 and the carrier medium was 10 mM Tris-HCl, pH 5.7 (Häussinger et al., 1983). Thin layer chromatography was then used to determine if any glutamate was present in the [1-^{14}C] glutamine preparation. The sample containing a known amount of radioactivity was spotted on the plate along with glutamine and glutamate standards. The plate was developed in n-propanol:concentrated ammonia (7:3 v/v). Spots were located by spraying with ninhydrin spray (0.2% in ethanol) and the sample was compared to the glutamine and glutamate standards as to the distance migrated. The sample spot was scraped for measurement of radioactivity. In all instances, less than 2% glutamate was found.

Animals

Male Sprague-Dawley rats (Charles River, Montreal) weighing 200-300g were used for all studies. Rats were

allowed water and Purina rat chow ad libitum.

Male Sprague-Dawley rats were made diabetic by a single injection of streptozotocin into the tail vein under light ether anaesthesia. The dose given was 100 mg/kg body wt as previously reported (Brosnan et al., 1983). The streptozotocin was made up at a concentration of 60 mg/ml of sodium citrate buffer, pH 4.5 and injected immediately. Insulin treatment was started 24 hours after the streptozotocin injection. Diabetic rats were maintained on Protamine Zinc insulin (PZI) for five days. Daily subcutaneous insulin injections were given between 13:00-14:00 hours. A control group of insulin-maintained diabetic rats were kept on insulin up to the day of experimentation. Blood glucose levels were monitored by obtaining a drop of blood from the tail vein and determining blood glucose by using an Ames Glucometer GX (Miles Canada Inc., Etobicoke, Ontario). Insulin dosages were adjusted to maintain normal blood glucose (4-6 mM) levels and weight gain. A blood sample from the portal vein was taken on the day of the experiment for later determination of plasma glucose levels. Plasma insulin and glucagon levels were not determined.

METHODS

Preparation of Hepatocytes

Hepatocytes were prepared essentially as described previously (Krebs et al., 1974) except that hyaluronidase was omitted from the perfusate. Rats were anaesthetized with pentabarbitol. The femoral vein was exposed and 0.1 ml of heparin was injected. The isolation of hepatocytes involved perfusing rat liver first with 500 ml of calcium-free Krebs-Henseleit bicarbonate medium containing 2 mM EGTA, 20 mM glucose, 2.1 mM lactate, and 0.3 mM pyruvate. The flow rate in all cases was approximately 40 ml/minute, all media were gassed with 95:5 O_2:CO_2 for 20 minutes prior to usage and gassing continued throughout the isolation procedure. This was followed by 500 ml of Krebs-Henseleit medium which contained 2.5 mM calcium, 20 mM glucose, 2.1 mM lactate, and 0.3 mM pyruvate. Calcium-containing Krebs-Henseleit medium with 0.25% bovine serum albumin (fraction V essentially fatty acid free) and 15 mg collagenase/100 ml was then recirculated for 15 to 20 minutes in a total volume of 200 ml. After this recirculation of 15 to 20 minutes, the liver began to leak medium and was observed to break apart, and at this point the liver was removed and minced in a petri dish. This suspension was then shaken in a Dubnoff metabolic shaker at 37°C under

O_2/CO_2 (95:5) for ten minutes. The cells were then spun down at 600 rpm and washed two more times in Krebs-Henseleit medium. The final wash was in Krebs-Henseleit medium containg 2.5% BSA and the final suspension of cells was also in this medium. Cell viability was determined by 0.2% trypan blue exclusion and was greater than 95% in all instances. Cells were quantified by determining their dry weight. A 3 ml aliquot of cell suspension was placed in a metal weighing pan and a 3 ml aliquot of the 2.5% BSA resuspension medium was placed in a separate weighing pan. Both were dried in an oven at 50°C for 24 hours. The difference in weight of the cell suspension and the medium was used to determine the dry weight of the cells present.

For the studies on streptozotocin-diabetic rats, a calcium concentration of 1.25 mM was used in the perfusions but the cells were still resuspended in 2.5 mM calcium Krebs-Henseleit medium with 2.5% BSA. This stemmed from initial studies where the calcium concentration was found to affect the overall success of the perfusion. When the perfusion medium was switched from calcium-free to 2.5 mM calcium-containing medium, the flow was observed to immediately slow down and eventually stop completely. Using 1.25 mM calcium prevented this and allowed for the isolation of viable cells.

Preparation of Mitochondria

Mitochondria were isolated from livers of male Sprague-Dawley rats as described previously (Jois et al., 1989). Rats were killed by cervical dislocation and the liver was quickly removed. The liver was homogenized, using a Potter-Elvejhem hand-held homogenizer, in an ice-cold medium containing 0.225 M mannitol, 0.075 M sucrose, 5 mM HEPES, and 1 mM EGTA. The homogenate was centrifuged for 10 minutes at 600 x g and the supernatant was then centrifuged at 8200 x g for 10 minutes. The resulting pellet was resuspended in the above medium and centrifuged at 8200 x g for 10 minutes. The resuspension and centrifugation steps were repeated two times and the final pellet was resuspended in a small volume of the above medium. The respiratory control ratio was determined polarographically at 30°C with 10 mM α-ketoglutarate as substrate and was greater than 4 in all cases. The incubation medium contained 140 mM KCl, 5 mM Tris, 4 mM KH_2PO_4, 2.5 mM $MgCl_2$, and 1.5 mM EDTA and was adjusted to pH 7.4. A standard biuret procedure was used for determining the protein concentration of the mitochondrial suspension (Gornall et al., 1949). Bovine serum albumin was used as standard.

Preparation of Liver Mitochondria from Diabetic Rats

Liver mitochondria isolated from diabetic rats using standard procedures were found to have poor respiratory control ratios. This has been reported by other investigators for both alloxan-induced diabetes (Harano et al., 1972; Lerner et al., 1972) and streptozotocin-induced diabetes (Brignone et al., 1982; Grinblat et al., 1988). This has been attributed to decreased state 3 respiration due to an inhibition of adenine nucleotide penetration (Harano et al., 1972; Lerner et al., 1972). This may be a consequence of increased hepatic lipid content, particularly long chain acyl-CoA esters, which occurs in diabetes and is known to inhibit the adenine nucleotide translocase (Lerner et al., 1972). Similar results have been found during hibernation and exhaustive exercise (Lerner et al., 1972; Klug et al., 1984; Soball et al., 1985; Bode et al., 1990).

The sluggish response to ADP in liver mitochondria may be partially reversed by addition of high concentrations of bovine serum albumin to the isolation medium (Lerner et al., 1972). Similar procedures have been used to reverse the poor response in liver mitochondria isolated from rats previously exposed to exhaustive exercise (Klug et al., 1984; Bode et al., 1990).

We devised a procedure which permitted us to isolate well-coupled mitochondria from streptozotocin-diabetic rats. The key point was to flush the liver with a mitochondrial homogenization medium that contained a high concentration of serum albumin. Thus the extracellular space was replaced with this medium so that, upon homogenization, lipids were efficently complexed. To isolate liver mitochondria from streptozotocin diabetic rats, the rats were anaesthetized with pentabarbitol. The femoral vein was exposed and 0.1 ml (100 units) of heparin injected. The body cavity was opened to expose the liver. Ties were placed around the hepatic artery and the portal vein. In order to reduce the fatty acid content of the liver, it was first washed with 50 ml of mitochondrial isolation medium containing 50 mg bovine serum albumin (fraction V, essentially fatty acid free BSA)/ml through a portal vein cannula. The medium was equilibrated at 37°C before use. A 50 ml syringe containing the medium was attached to the cannula, the hepatic artery was tied off and the vena cava cut. The liver was then slowly flushed with the BSA-containing isolation medium. Immediately the liver was removed and placed in ice-cold isolation medium containing 5 mg BSA/ml. The liver was homogenized in this medium. The centrifugation and resuspension steps were the same as the

standard mitochondrial isolation procedure except that all media contained 5 mg BSA/ml. The respiratory control ratio was determined using the previously stated procedure. Livers from control, insulin-maintained diabetic, and diabetic rats were subjected to this procedure.

Preparation of Mitochondrial Membranes

Glutaminase assays were carried out on mitochondrial membranes prepared as described by Szweda and Atkinson (1990a). This involved preparing mitochondria using the standard isolation procedure. The mitochondrial pellet was resuspended in isolation medium (0.225 M mannitol, 0.075 M sucrose, 5 mM Hepes, and 1 mM EGTA), and then diluted 1:1 with distilled water. The mitochondria were disrupted by freezing in liquid nitrogen and were then thawed in water at 37°C. This freezing and thawing was repeated three times (Szweda and Atkinson, 1990a). The suspension was diluted 1:4 with mitochondrial isolation medium and this was followed by centrifugation at 40,000 x g for 20 minutes to isolate the membrane fraction. The pellet was resuspended in mitochondrial isolation medium and adjusted to a final protein concentration of 20 mg/ml.

Isolation of Mitochondria from Hepatocytes

Hepatocytes were isolated from the livers of fed rats as previously indicated. These hepatocytes were incubated at 37°C under O_2/CO_2 (95:5) in a shaking water bath with 10^{-7}M glucagon (dissolved in 10 mM HCl) or 1 μM okadaic acid (dissolved in DMSO). Control incubations contained an equivalent amount of 10 mM HCl or DMSO, respectively. All incubations were at a final volume of 20 ml in 50 ml Erlenmeyer flasks and were gassed with 95:5 O_2:CO_2. The incubation was terminated by spinning the hepatocytes at 600 rpm for 2 minutes to remove the Krebs-Henseleit medium. A modification of a procedure used by Covera and Garcia-Sáinz (1983) was used to obtain the mitochondria. In this method digitonin is used to disrupt the plasma membranes and permit the release of mitochondria. The cells were resuspended in isolation medium containing 0.225 M mannitol, 0.075 M sucrose, 5 mM HEPES, 1 mM EGTA and 0.4 mg digitonin/ml. The cells were subjected to a high speed spin (8200 x g) for two minutes. The cell pellet was homogenized in digitonin-free isolation medium using ten strokes of a Potter-Elvejhem hand-held homogenizer. The suspension was then subjected to the same procedure as used for the isolation of mitochondria from intact liver. Again, the respiratory control ratio was

checked using a Clark oxygen electrode with 10 mM α-ketoglutarate as substrate and was greater than 3 in all cases.

Measurement of flux through glutaminase in isolated hepatocytes

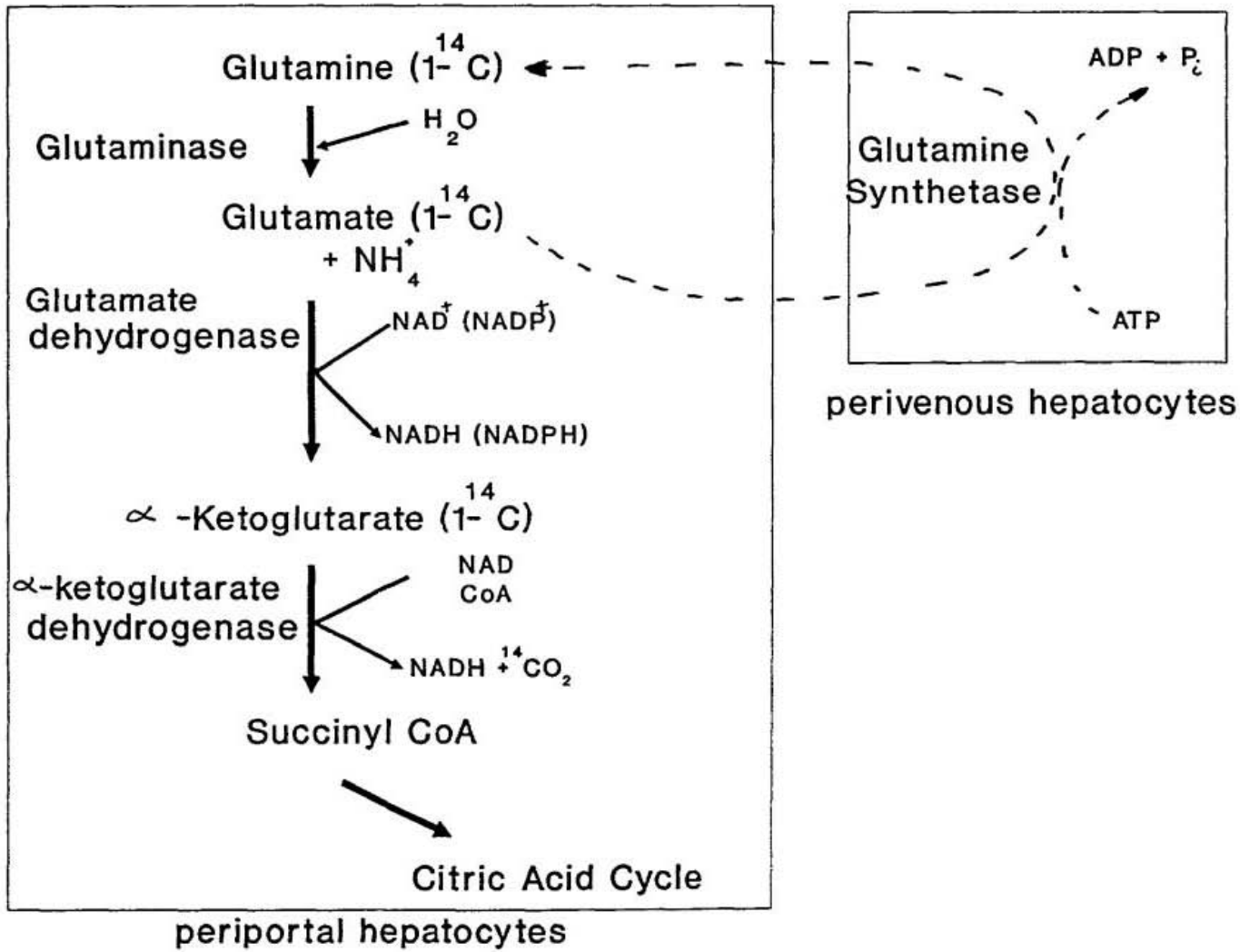
The procedure for determining flux through glutaminase involves the trapping and counting of $^{14}CO_2$ after incubation of cells with [1-^{14}C]glutamine (1 mM) (Vincent et al., 1989). Incubations were in triplicate in 25 ml Erlenmeyer flasks containing between 4-6 mg dry weight of hepatocytes in a final volume of 1 ml. Cells were preincubated in Krebs-Henseleit medium for 20 minutes at 37°C before the addition of [1-^{14}C]glutamine to give a final concentration of 1 mM. Where indicated, ammonium chloride was added at the same time as the [1-^{14}C]glutamine. Each flask was gassed with 95:5 O_2:CO_2 for 20 seconds after the addition of the cells and also after addition of [1-^{14}C]glutamine. The incubation flasks were equipped with rubber septa in which plastic centre wells were suspended. NCS tissue solubilizer was introduced into centre wells through the septa just before termination of incubation with 0.15 ml of 30% (w/v) perchloric acid. $^{14}CO_2$ was collected for one hour and the centre wells were then transferred to

scintillation vials containing 15 ml of scintillation fluid (Omnifluor) and radioactivity was determined.

There are two potential problems with using CO_2 release to measure flux through glutaminase. The first stems from the fact that there are two steps between glutaminase and the actual release of CO_2 by α-ketoglutarate dehydrogenase. Thus CO_2 release is somewhat removed from the actual enzyme being measured and there is opportunity for isotope dilution to occur. The second complication arises because the isolated hepatocyte suspension contains periportal cells with glutaminase and perivenous cells with glutamine synthetase. Thus glutamate, produced by glutaminase, could be resynthesized into glutamine by glutamine synthetase without release of $^{14}CO_2$ (see Figure 2.1). This would underestimate the extent of glutamine utilization by glutaminase. Methionine sulfoximine (MSO), however, can be used as an inhibitor of glutamine synthetase (Meister, 1968).

To test the validity of using $^{14}CO_2$ release from [1-^{14}C]glutamine to determine glutaminase flux, glutamine disappearance was determined chemically and $^{14}CO_2$ was simultaneously collected both in the presence and absence of 1 mM methionine sulfoximine (Vincent et al., 1989). This procedure will determine whether glutamine disappearance in

Figure 2.1. Illustration of Potential Problems of Using $^{14}CO_2$ Release from [1-^{14}C] Glutamine to Determine Flux Through Glutaminase in Isolated Hepatocytes


Glutamine (1- ^{14}C)
H_2O
Glutaminase
Glutamate (1- ^{14}C)
+ NH_4^+
Glutamate dehydrogenase
NAD^+ ($NADP^+$)
NADH (NADPH)
α -Ketoglutarate (1- ^{14}C)
α-ketoglutarate dehydrogenase
NAD
CoA
NADH + $^{14}CO_2$
Succinyl CoA
Citric Acid Cycle
periportal hepatocytes
ADP + P_i
Glutamine Synthetase
ATP
perivenous hepatocytes

the presence of methionine sulfoximine was equal to $^{14}CO_2$ release and also whether methionine sulfoximine was necessary for the [1-^{14}C]glutamine assay.

Each 25 ml Erlenmeyer flask contained 3 ml of Krebs-2Henseleit medium with approximately 45 mg dry weight cells/flask. The flasks were gassed with 95:5 O_2:CO_2 and the hepatocytes were preincubated for 20 minutes at 37°C. The reaction was started by adding 1 mM [1-^{14}C]glutamine, 2 mM NH_4Cl, and, where indicated, 10^{-7} M glucagon to the flasks. Immediately a 1 ml sample was taken and added to 0.2 ml of 30% perchloric acid for glutamine and glutamate determinations in these zero time or initial samples. After 30 minutes, 1 ml samples were removed from the flasks and added to perchloric acid for glutamine and glutamate measurements. The incubation of the remaining 1 ml was stopped by adding 0.2 ml of 30% perchloric acid to the flasks. $^{14}CO_2$ was trapped as previously described. Glutamine was determined enzymatically by employing glutaminase and glutamate dehydrogenase (Lund, 1974). Glutamate was determined using the glutamate dehydrogenase method (Bernt & Bergmeyer, 1974).

Table 2.1 gives glutamine disappearance, glutamate formation and $^{14}CO_2$ production. In this study a concentrated cell suspension was used since more cells were required in

Table 2.1. Glutamine Disappearance and $^{14}CO_2$ Release in Isolated Hepatocytes[a]

Values are Mean ± SEM for the number of experiments indicated in parentheses, and are expressed as nmoles/ mg dry wt./min. The concentrations used are 1 mM 1-^{14}C-Glutamine, 2 mM ammonium chloride, 10^{-7}M glucagon, and 1 mM methionine sulfoximine. Normal, non-diabetic rats were used.

(* P<0.05 vs no added glucagon, # P<0.05 vs no added MSO).

Additions	Glutamine disappearance	$^{14}CO_2$ release	Glutamate formation	Glutamine disappearnce / $^{14}CO_2$ release
none	-0.062 ± 0.09 (5)	0.19 ± 0.01 (5)	0.11 ± 0.01 (5)	-
MSO	0.18 ± 0.04# (5)	0.20 ± 0.01 (5)	0.15 ± 0.02 (5)	0.89
Glucagon	0.12 ± 0.05 (4)	0.35 ± 0.02* (4)	0.047 ± 0.01* (4)	0.34
Glucagon + MSO	0.25 ± 0.03 (3)	0.32 ± 0.01* (4)	0.068 ± 0.01* (4)	0.79

[a] Experiment completed with H. Stephen Ewart and is also contained in his Ph.D. Thesis.

order to accurately determine changes in glutamine and glutamate. This cell concentration was also used by other investigators (Vincent et al., 1989). The results of Table 2.1 indicate that in the absence of added MSO, glutamine disappearance was not found but $^{14}CO_2$ release did occur. This suggests that glutamine disappearance determined enzymatically in the absence of MSO is underestimating glutaminase flux due to the production of glutamine by glutamine synthetase in the perivenous hepatocytes. In the presence of 1 mM MSO, glutamine disappearance was the same as $^{14}CO_2$ release (0.18 ± 0.04 vs 0.20 ± 0.01 nmoles/mg dry weight cells/min.). It should also be noted that $^{14}CO_2$ release was unaffected by the presence of MSO. Thus $^{14}CO_2$ release does represent a valid method for determining flux through glutaminase.

Glycogenolysis in isolated hepatocytes

Glycogenolysis was determined in hepatocytes isolated from fed rats using the amount of glucose produced over a 30 minute time period. Incubations were in triplicate with a final volume of 1 ml in 25 ml Erlenmeyer flasks. The incubations were in 2.5 mM Ca^{2+}-containing Krebs-Henseleit medium, gassed with 95:5 O_2:CO_2, and where indicated Rp-cAMPS was added with the cells and subjected to a 20 minute

preincubation at 37°C before the addition of Sp-cAMPS or glucagon. The amount of glucose formed during this preincubation was subtracted in the calculation of the glucose formed during the 30 minute incubation. After 30 minutes at 37°C in a Dubnoff metabolic shaker, the reactions were stopped by adding 0.15 ml of 30% PCA (w/v). The contents of the flask were poured into Eppendorf tubes and centrifuged at 12,000 g for 2 minutes. The supernatant was neutralized with 3 M K_3PO_4 and glucose was assayed in the supernatant using a standard method (Bergmeyer et al., 1974).

Glutaminase Flux in Isolated Mitochondria

Isolated mitochondria were incubated in 25 ml Erlenmeyer flasks for 10 minutes at 30°C. All incubations were in triplicate and each flask contained 2-3 mg mitochondrial protein. The incubation medium contained 0.1 M KCl, 10 mM Tris, 10 mM K_2HPO_4, 4 mM succinate, 10 mM $KHCO_3$, 13 μM rotenone, 1 mM EGTA, and 20 mM glutamine (Lacey et al., 1981). After 10 minutes, the reaction was stopped with 0.3 ml of perchloric acid (7% w/v). For zero time samples 0.3 ml of perchloric acid was added before the mitochondria. The protein was then removed by centrifugation in an Eppendorf microcentrifuge for 2 minutes at 12,000 x g. The supernatant

was taken and adjusted to pH 8.8 with 3 M K_3PO_4 and was then used for the glutamate assay (Bernt and Bergmeyer, 1974). Glutamate formation was calculated as the difference between the total flask contents at zero time and after the 10 minute period of incubation. Since rotenone was present to prevent further glutamate oxidation, the amount of glutamate formed was a measure of flux through glutaminase.

Measurement of Glutaminase Activity in Mitochondrial Membranes

The membranes isolated from freeze-thawed mitochondria were incubated at 37°C in 300 mM mannitol, 10 mM Hepes, 20 mM glutamine, 0.7 mM NH_4Cl and various concentrations of phosphate at pH 7.4 (Szweda and Atkinson, 1990a). The reaction was started by adding 50 μl of 20 mg/ml of membranes to give a final protein concentration of 1 mg/ml. The reaction was terminated after 10 minutes by adding 0.3 ml of 7% (w/v) perchloric acid. The supernatant was adjusted to pH 8.8 with K_3PO_4 and glutamate was determined using a glutamate dehydrogenase assay (Bernt and Bergmeyer, 1974).

Portal plasma glucose concentration

Rats were anaesthetized with pentabarbitol and a blood sample was withdrawn from the portal vein using a heparinized

syringe. This blood sample was placed in an Eppendorf centrifuge tube and centrifuged for 2 minutes, (12,000 g, 5°C), to separate the plasma. The plasma sample was stored at -20°C for later determination of plasma glucose using a standard method (Bergmeyer et al., 1974).

Statistical Analysis

All values are expressed as Mean ± SD or Mean ± SEM as indicated. Statistical analysis was by student's t-test or paired t-test as indicated. Multiple comparisons were tested using ANOVA and the Tukey multiple comparison test.

CHAPTER 3
RESULTS AND DISCUSSION
DIABETES MELLITUS AND GLUTAMINE CATABOLISM

INTRODUCTION

Diabetes mellitus represents a physiological disorder in which hyperglucagonemia is a major characteristic. Glutamine can serve as a precursor for glucose synthesis in the liver. Hepatic glutaminase is known to be regulated by glucogenic hormones such as glucagon. Stemming from these facts, a number of questions arise regarding hepatic glutamine catabolism in diabetes mellitus. These questions will be examined using streptozotocin-induced diabetes as a model.

RESULTS

Portal plasma glucose levels and body weights

Rats were made diabetic by a single injection of streptozotocin into the tail vein. The dose given was 100 mg streptozotocin/kg body weight. Blood glucose levels were determined by obtaining a drop of blood from the tail vein and using an Ames Glucometer GX. Twenty-four hours after the streptozotocin injection, blood glucose levels were greater than 12 mM thus confirming the induction of diabetes. Diabetic rats were maintained on daily subcutaneous insulin injections for five days and then insulin was withheld for five days preceeding the day of the experiment. A control group received daily insulin injections until the day of the

experiment. A third, non-diabetic control, group was also used. On the day of the experiment, the rats were weighed and a blood sample was taken from the portal vein. The portal plasma glucose levels and body weights are given in Table 3.1. These values are for the actual day of the experiment. The hepatic portal vein was chosen since it is the major blood supply to the liver. These results show that insulin treatment of diabetic rats for approximately ten days brings the portal plasma glucose levels back to normal, non-diabetic levels. The portal plasma glucose in the diabetic rats was significantly higher than in the normal and the insulin-maintained diabetic animals. The body weight was also significantly lower in this group.

Flux through glutaminase in isolated hepatocytes

The first question to be answered is whether streptozotocin-induced diabetes results in increased flux through glutaminase in isolated hepatocytes. Connected to this question is whether daily insulin treatment of diabetic rats could prevent any increase observed. Therefore, flux through glutaminase in hepatocytes isolated from the three different groups of animals was examined. Since ammonia is an activator of glutaminase, three different ammonia

Table 3.1 Portal Plasma Glucose Levels and Body Weights

Values given are mean ± SEM for the number of animals indicated. Glucose was enzymatically determined using the glucose-6-phosphatase dehydrogenase/hexokinase method.

* $P<0.05$ vs. normal and insulin-maintained.

Rat Type	Portal Plasma Glucose (mM)	Body Wt. (g)
Control, non-diabetic (12)	9.1 ± 0.6	340 ± 13
Insulin-Maintained Diabetic (11)	8.0 ± 1.5	330 ± 6
Diabetic (12)	29.2 ± 1.7*	298 ± 9*

concentrations were used in these studies. Glutaminase flux in isolated hepatocytes was determined by measuring $^{14}CO_2$ produced from [1-^{14}C]glutamine in the absence of MSO. This method was validated in **Chapter 2** as an accurate method of measuring glutaminase flux.

Table 3.2 gives data on the flux through glutaminase in isolated hepatocytes from control, insulin-maintained diabetic, and diabetic rats. With no added ammonium chloride, flux through glutaminase is low for the three groups although the flux is higher in the diabetic group compared to the insulin-maintained group. At a near-physiological portal vein concentration of 0.5 mM ammonium chloride, flux through glutaminase in the hepatocytes isolated from the diabetic rats is significantly higher than in hepatocytes from the non-diabetic controls or insulin-maintained diabetic. Thus diabetes stimulated flux through glutaminase and daily insulin treatment prevented the increase in flux in these isolatd hepatocytes. At 2 mM added ammonium chloride, glutaminase flux is increased in the three groups and there is no significant difference among the three although the significant trend seen at 0.5 mM NH_4Cl is still evident.

The glucogenic hormone glucagon has been shown to stimulate flux through glutaminase in perfused rat liver

Table 3.2 Flux Through Glutaminase in Isolated Hepatoytes from Control, Insulin-maintained Diabetic, and Diabetic Rats

Glutaminase flux in isolated hepatocytes as determined by $^{14}CO_2$ release. Values are mean ± SEM; n=4 for control and diabetic, n=3 for insulin-maintained diabetic. Final concentrations: glucagon 0.1 μM, epinephrine 1.0 μM, [1-^{14}C]Glutamine 1 mM. Each flask contained between 4-6 mg dry weight of hepatocytes. Rats were made diabetic with an injection of 100 mg streptozotocin/kg body weight. (a,b represent $p<0.05$ vs. control and insulin-maintained, respectively, using the Tukey multiple comparison test. * $P<0.05$ vs no added hormone at NH_4Cl concentration indicated; paired t-test)

	Control	Insulin-maintained	Diabetic
No Addition	5.55 ± 0.64	3.56 ± 0.34	7.10 ± 0.65[b]
+ Glucagon	5.62 ± 0.64	5.19 ± 0.33	8.03 ± 0.72[a,b]
+ Epinephrine	8.10 ± 0.69	6.57 ± 0.94	11.58 ± 1.64[b]
+ 0.5 mM NH_4Cl	6.98 ± 1.28	5.43 ± 1.58	13.74 ± 2.09[a,b]
+ Glucagon	9.58 ± 0.60	12.63 ± 0.60[a,*]	19.85 ± 3.41[a,*]
+ Epinephrine	13.73 ± 0.95[*]	19.02 ± 1.10[a,*]	30.67 ± 4.70[a,*]
+ 2 mM NH_4Cl	19.67 ± 3.11	21.70 ± 2.69	33.20 ± 5.87
+ Glucagon	39.57 ± 4.20[*]	40.00 ± 4.52[*]	45.73 ± 4.74[*]
+ Epinephrine	45.93 ± 3.66[*]	37.27 ± 3.71[*]	61.02 ± 9.37[*]

(Häussinger et al., 1983) and in isolated hepatocytes (Covera and García-Sáinz, 1983). Glucose production in isolated hepatocytes with glutamine as the substrate is also increased with glucagon (Joseph et al., 1978). It has been previously shown that glucagon, and the catecholamine epinephrine, increase flux through the glycine cleavage enzyme system in isolated hepatocytes (Jois et al., 1989). This enzyme system, like glutaminase, is located within the mitochondria. Thus the third question examined is whether glucagon and epinephrine stimulate glutaminase flux in isolated hepatocytes and, if so, are there any differences among the three groups. Therefore, as indicated in Table 3.2, the effects of glucagon and epinephrine on glutaminase flux in isolated hepatocytes was examined. In the absence of added ammonium chloride there was no stimulation by either glucagon or epinephrine. In the presence of 0.5 mM added ammonium chloride, epinephrine stimulated flux in the isolated hepatocytes from the three groups. Significant glucagon stimulation of glutaminase flux occurred in hepatocytes isolated from the insulin-maintained diabetic and the diabetic rats. The pattern of stimulation is similar in hepatocytes from control and diabetic rats. Both glucagon and epinephrine stimulated glutaminase flux to a greater extent in hepatocytes isolated from insulin-maintained

diabetic rats than in those from control and diabetic rats. The reason for this is unclear but it may be related to possible alterations in the insulin:glucagon ratio caused by the subcutaneous injections of insulin. This would also account for the lower basal rates observed with the hepatocytes from the insulin-maintained diabetics. Both glucagon and epinephrine stimulated glutaminase flux at 2 mM added ammonium chloride.

Glutaminase Flux in Isolated Mitochondria

A fourth question examined is whether glutaminase flux is stimulated in mitochondria isolated from livers of diabetic rats. There was, initially, a problem in addressing this question. Using standard differential centrifugation procedures, liver mitochondria isolated from diabetic rats have been found to have poor respiratory control ratios (Lerner et al., 1972; Grinblat et al., 1988). This has been attributed to decreased state 3 respiration due to an inhibition of adenine nucleotide penetration possibly caused by an increased hepatic lipid content. It was therefore decided to flush the liver with a mitochondrial homogenization medium that contained a high concentration of serum albumin in an attempt to complex any lipids present. Using this BSA

perfusion technique, liver mitochondria were isolated from control, insulin-maintained diabetic, and diabetic rats. The respiratory control ratios were not different among the three groups and indicate that the mitochondria are well coupled.

It was established by Lacey et al. (1981) that glutaminase flux in intact mitochondria from glucagon-injected rats was more sensitive to activation by inorganic phosphate (i.e. the phosphate activation curve is left-shifted). This was not evident, however, when glutaminase was assayed in broken mitochondria. A similar phenomenon has been reported by Ewart and Brosnan (1993) for glutaminase activated after protein feeding. Therefore to determine whether diabetes stimulates flux through glutaminase, three different added phosphate concentrations were used in the incubations (Figure 3.1). At all phosphate concentrations used, glutaminase flux in the mitochondria from the diabetic rats was significantly higher than in mitochondria from control, non-diabetic rats. Flux through glutaminase in the mitochondria isolated from the insulin-maintained diabetic rats was not as high as that from the diabetics and was not significantly higher than in the control, non-diabetics as is indicated in Figure 3.1. Although no significant differences were found, the flux in the mitochondria from the insulin-maintained rats does seem to

be higher than that obtained in the non-diabetic controls. Insulin treatment of streptozotocin diabetic rats may not return the plasma glucagon levels to normal, non-diabetic values (Patel, 1983; Brubaker et al., 1991). Such an increase in circulating glucagon levels may account for the higher glutaminase flux. The insert to Figure 3.1 gives the ratio of glutaminase flux determined at 20 mM phosphate compared to that at 2.5 mM phosphate. This ratio is significantly higher for the control, non-diabetics compared to the diabetics. This indicates that, similar to the results with the glucagon-injected rats, there is some left-shifting of the phosphate curve for glutaminase. The hyperglucagonemic situation found in diabetes mellitus could, possibly, also be increasing the sensitivity of glutaminase for inorganic phosphate.

There are, however, other possible reasons for this increased glutaminase flux. One such possibility is that there is more ammonia present in the mitochondrial preparation prepared from the diabetic rats. Glutaminase is clearly stimulated by its product, ammonia, and if there were more ammonia associated with the diabetic mitochondria then this could account for the increase in glutaminase flux observed. To test this, glutaminase flux was determined in the presence of 10 mM phosphate and a maximal stimulatory NH_4Cl

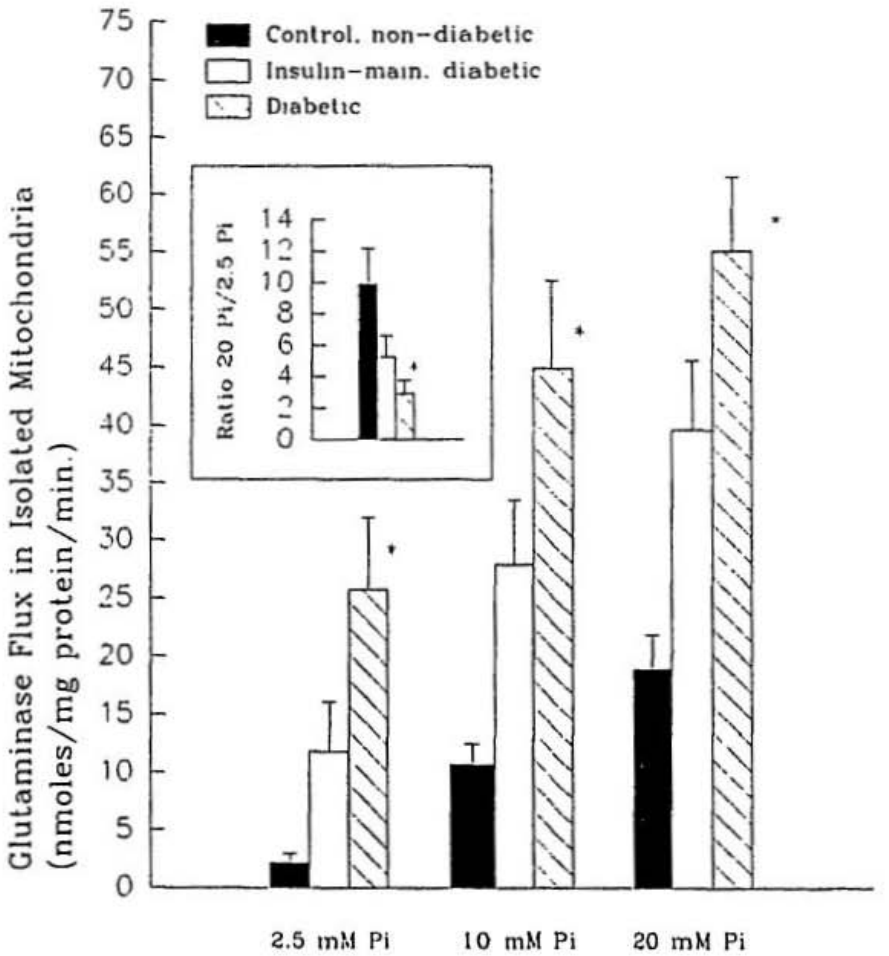
concentration of 1 mM. Under these conditions, glutaminase flux was still observed to be higher in the mitochondria isolated from the diabetic rats (16.5 nmoles/min per mg protein for control vs. 44.6 nmoles/min per mg protein for diabetic; average of two experiments). This indicates that the increase in flux observed in the diabetics can not be due to more ammonia being present in the diabetic mitochondrial preparation since even in the presence of a maximal stimulatory added ammonia concentration the increase associated with diabetes is still observed.

Glutaminase activity determined using mitochondrial membranes

Lacey et al. (1981) found that the left-shift of the phosphate curve seen in intact mitochondria from glucagon-injected rats was not seen when examined in broken mitochondria. In fact, there was no difference in glutaminase activity at any phosphate concentration tested. In this particular situation involving glucagon-injected rats, the mitochondria were isolated twenty minutes after the injection. In this short amount of time it is unlikely that there is an increase in the amount of glutaminase protein. With streptozotocin-induced diabetes, however, there are ten days

Figure 3.1 Glutaminase Flux in Isolated Mitochondria

Liver mitochondria were isolated using the BSA perfusion method as indicated in **Chapter 2.** Glutaminase flux was determined using 20 mM glutamine and the indicated phosphate concentration. The insert shows the ratio of glutaminase flux at 20 mM phosphate compared to flux at 2.5 mM phosphate. The respiratory control ratios were determined in the presence of 10 mM α-ketoglutarate and were 4.2 ± 0.3, 4.6 ± 0.3, and 4.2 ± 0.2, for control, insulin-maintained, and diabetic, respectively. Values are mean ± SEM; n=4. (* $P<0.05$ vs control).


Control, non-diabetic
Insulin-main. diabetic
Diabetic
Glutaminase Flux in Isolated Mitochondria
(nmoles/mg protein/min.)
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
Ratio 20 Pi/2.5 Pi
14
12
10
8
6
4
0
2.5 mM Pi
10 mM Pi
20 mM Pi

between the induction of diabetes and the actual determination of glutaminase flux. Therefore with diabetes it is possible that there is an increase in total glutaminase activity due to more glutaminase protein being present. Thus glutaminase activity was determined using mitochondrial membranes prepared from freeze-thawed mitochondria. Two differnt added phosphate concentrations were used, 10 mM and 40 mM. As is indicated in Table 3.3, glutaminase activity is increased in the diabetic rats. Maintaining the diabetics on insulin prevented this increase. Similar results have been reported for glutaminase activity in liver homogenates from streptozotocin diabetic rats (Watford et al., 1984). This study also found that insulin treatment of diabetic rats prevented the increase in glutaminase activity.

An interesting result in Table 3.3 shows that when the ratio of glutaminase activity at 40 mM phosphate is compared to that at 10 mM phosphate, it is 2 for both the control and the insulin-maintained diabetic. This ratio was, however, approximately 1.5 for the diabetics. Initially I thought that this was an error of some sort. Therefore, the experiment was repeated for the streptozotocin-induced diabetic group. The results were essentially the same. The ratio of glutaminase activity at 40 mM phosphate compared to 10 mM phosphate was

found to be 1.6 (97.9 ± 10.3 vs. 59.6 ± 5.8 nmoles/min per mg protein; n=3). Thus it appears that there is a slight change in the sensitivity of glutaminase for phosphate even when assayed in broken mitochondria from diabetic rats. This requires more investigation but it is conceivable that a stable change in glutaminase properties in diabetes may have been uncovered.

Table 3.3 Glutaminase Activity in Mitochondrial Membranes

Glutaminase activity was determined, using mitochondrial membranes prepared from freeze-thawed mitochondria, in the presence of 20 mM glutamine, 0.5 mM NH_4Cl, and the phosphate concentrations indicated. Values given are nmoles glutamate formed/mg protein per minute and are mean ± SEM for the indicated number of preparations. * $P<0.05$ vs. control and insulin-maintained.

Added Phosphate	Control, non-diabetic (4)	Insulin-maintained diabetic (4)	Diabetic (4)
10 mM	28.7 ± 3.7	32.6 ± 4.5	80.6 ± 5.8*
40 mM	56.0 ± 4.3	64.3 ± 4.5	123.4 ± 8.6*

DISCUSSION

Diabetes mellitus is characterized by a decrease in or total lack of insulin action and a relative increase in counter regulatory hormones such as glucagon. A major problem associated with diabetes is continued production of glucose by gluconeogenesis even in the presence of hyperglycemia. Glutamine, being a glucogenic amino acid, can be converted to glucose in the liver. The first step in this conversion involves the formation of glutamate from glutamine, a conversion catalysed by the mitochondrial enzyme glutaminase.

Hepatic glutaminase is regulated by hormones (Joseph and McGivan, 1978a; Häussinger et al., 1983; Table 3.2). Associated with diabetes mellitus is an increase in glutaminase activity due to more glutaminase protein (Watford et al., 1984; Smith and Watford, 1988). Previously, however, little attention has been given to the involvement of hormonal changes in the regulation of glutaminase in diabetes mellitus. This was examined in more detail using both isolated hepatocytes and isolated mitochondria from streptozotocin-induced diabetic rats. The first question examined dealt with glutaminase flux in isolated hepatocytes. The results of Table 3.2 indicate that glutaminase flux was increased in the hepatocytes from the diabetic group compared to the non-

diabetic control group. Maintaining the diabetic rats on daily insulin injections prevented the increase in glutaminase flux in isolated hepatocytes.

Liver mitochondria isolated from diabetic rats using conventional differential centrifugation techniques have traditionally had poor respiratory control ratios. This was solved in this study by flushing the liver with an homogenization medium containing a high concentration of BSA (50 mg/ml) and then isolating the mitochondria using a medium containing a lower concentration of BSA (5 mg/ml). It was believed that the poor respiratory ratios were due to high hepatic lipid content which inhibited adenine nucleotide penetration. BSA binds these lipids, enabling state 3 respiration to be increased.

Glutaminase flux was examined in these "well-coupled" mitochondria and it was found that the flux was significantly increased in mitochondria from diabetic rats (Figure 3.1). A major characteristic of glutaminase flux in mitochondria from glucagon-injected rats (Lacey et al., 1981) and from high-protein fed rats (Ewart and Brosnan, 1993) is an increased sensitivity of glutaminase to phosphate. As shown in Figure 3.1, the ratio of glutaminase flux determined at 20 mM phosphate compared to flux at 2.5 mM phosphate is much lower

for the diabetics compared to the control and the insulin-maintained groups. This clearly indicates an increased sensitivity of glutaminase for phosphate in the mitochondria from the diabetic rats and provides additional support for the involvement of glucagon in the observed increase in glutaminase flux.

The above results do not rule out the involvement of increased glutaminase activity in contributing to the increased glutaminase flux observed. Therefore, glutaminase activity was determined using mitochondrial membranes prepared from freeze-thawed mitochondria. The results of Table 3.3 indicate that, in agreement with previous published findings (Watford et al., 1984), glutaminase activity is increased with diabetes and insulin treatment of the diabetic rats prevents this increase. At 10 mM phosphate the glutaminase activity increased by approximately 2.8 fold whereas the glutaminase flux increased by 3.8 fold. Therefore the increased glutaminase flux in isolated mitochondria cannot be accounted for by only an increased glutaminase activity. Figure 3.1 also shows an increased sensitivity of glutaminase for phosphate in the mitochondria from the diabetic rats. These results indicate that glucagon is playing a significant role in increasing glutaminase flux in the isolated mitochondria.

The results obtained using the isolated hepatocytes can be accounted for by the increase in glutaminase activity. Previous studies with isolated hepatocytes and perfused rat liver have shown decreased glucagon sensitivities in these systems (Ewart, 1993). Therefore the hepatocyte results do not rule out the involvement of glucagon in controlling glutaminase in diabetes.

Maintaining the streptozotocin diabetic rats on daily insulin injections prevents or reverses the increase in glutaminase flux in isolated hepatocytes and isolated intact liver mitochondria. Insulin treatment also prevents the increase in glutaminase activity observed in mitochondrial membranes from freeze-thawed mitochondria. This lends support for the involvement of hormones in regulating hepatic glutaminase and strengthens the importance of the insulin/glucagon ratio in the regulation of gluconeogenesis.

Chapter 4
Results and Discussion
Hormonal Regulation of Glutaminase

INTRODUCTION

In the preceding section it was shown that the mitochondrial enzyme glutaminase is subject to hormonal control, being stimulated by glucagon and epinephrine. There is still the question, however, of exactly how the hormonal signal gets transmitted from the receptors on the plasma membrane through the cytosol to the mitochondria. This aspect of hormonal regulation will be examined in more detail in this chapter.

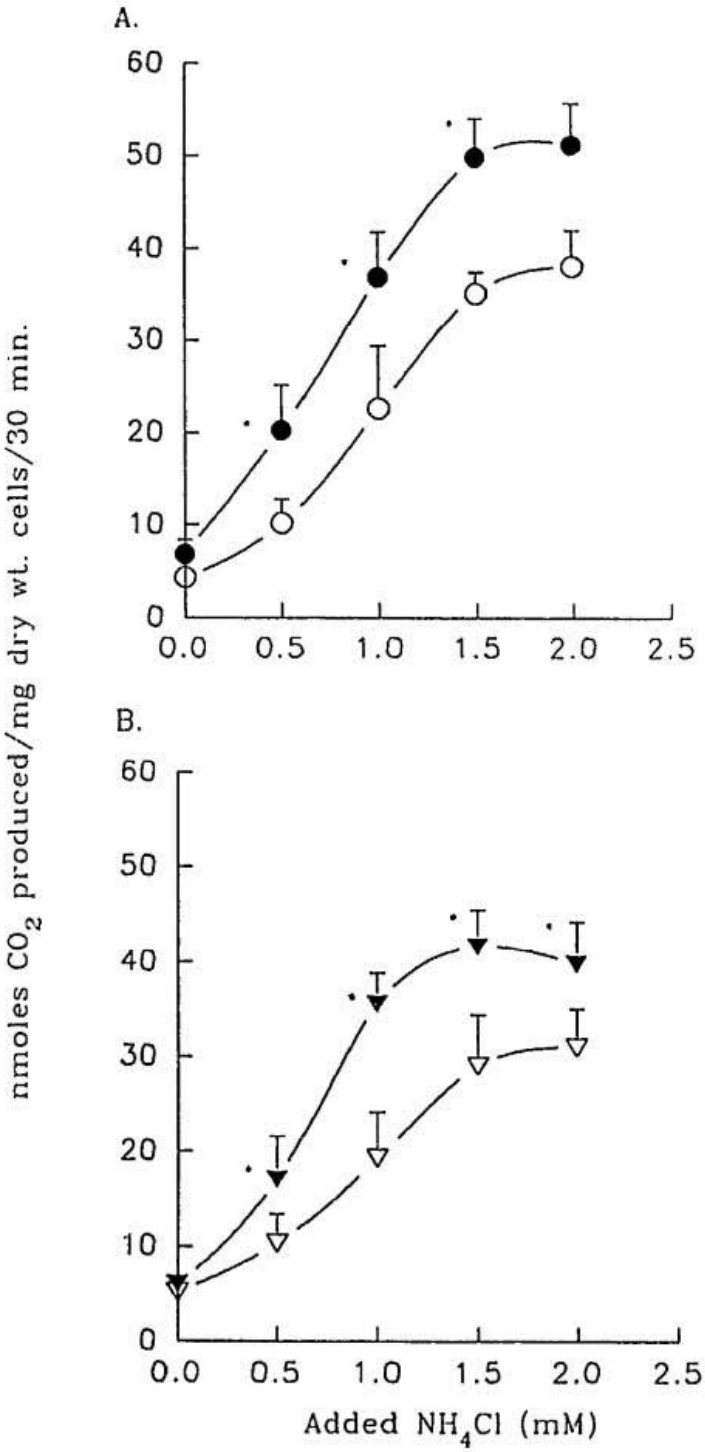
RESULTS

Glucagon and Epinephrine Stimulation of Glutaminase

Liver glutaminase has been shown to be under hormonal regulation. In Figure 4.1 glutaminase flux was determined in isolated hepatocytes in the presence of either epinephrine or glucagon as a function of ammonium concentration. These results again demonstrate that glutaminase is stimulated by ammonium chloride and also by the catecholamine epinephrine, and the glucogenic hormone glucagon. With no added ammonium chloride, there was no stimulation of glutaminase by either glucagon or epinephrine. In the presence of ammonium chloride, both hormones caused an increase in $^{14}CO_2$ release and thus in flux through glutaminase. In the presence of 1 mM NH_4Cl, 10^{-7}M vasopressin also stimulated glutaminase flux in

Figure 4.1. Effects of Epinephrine and Glucagon on Flux Through Glutaminase in Isolated Hepatocytes

Flux through glutaminase in isolated hepatocytes determined using 1 mM [1-^{14}C]glutamine and collecting $^{14}CO_2$. In A., epinephrine (●), 10^{-6}M, was added to the incubations while (O) represents the contrcl incubations. In B., glucagon (▾), 10^{-7}M, was added to the incubations while (▽) represents control incubations. Since both hormones were dissolved in 10 mM HCl, the control incubations contained an equivalent amount of 10 mM HCl (0.1 mM final concentration). Hepatocytes were preincubated at 37°C in Krebs-Henseleit bicarbonate medium for 20 minutes before the addition of [1-^{14}C] glutamine, hormones, and the indicated concentration of NH_4Cl. Values are nmoles CO_2 produced/mg dry wt. cells/30 min. and are mean ± SEM; n=4. *P<0.05 vs. no added hormone, paired t-test.


A.
B.
60
50
40
30
20
10
0
0.0
0.5
1.0
1.5
2.0
2.5
nmoles CO_2 produced/mg dry wt. cells/30 min.
Added NH_4Cl (mM)

isolated hepatocytes (16.8 ± 4.2 vs 31.4 ± 6.1 nmoles CO_2 produced/mg dry wt cells/30 min.).

Protein Phosphorylation and Glutaminase flux

A major unanswered question is exactly how and in what form the hormonal signal is transmitted to the mitochondria to affect enzymes such as glutaminase. The stimulation of glutaminase by hormones such as glucagon is quite stable, persisting in liver mitochondria isolated from rats previously injected with glucagon (Lacey et al., 1981). This differs from the effect of glucagon on the phosphorylation of key cytoplasmic enzymes where the phosphorylation is metabolically labile and can be reversed by phosphatases. The possibility does exist, however, that a phosphorylation step may be involved in the overall signalling process. To examine this further, the cytosolic protein phosphatase inhibitors okadaic acid and calyculin A were used. As is indicated in Figure 4.2 both of these phosphatase inhibitors caused a significant increase in glutaminase flux in isolated hepatocytes. The maximal effect was observed at 1 μM added okadaic acid or calyculin A. In subsequent experiments only okadaic acid was used since there was no significant difference between the two phosphatase inhibitors tested. Figure 4.3 indicates that okadaic acid causes a slight stimulation in the absence of added NH_4Cl but this stimulation

Figure 4.2. Effects of Protein Phosphatase Inhibitors on flux through Glutaminase in Isolated Hepatocytes

Glutaminase flux in isolated heptocytes was determined in the presence of 1 mM [1-^{14}C]glutamine and 1 mM added NH_4Cl. Hepatocytes were preincubated for 20 minutes at 37°C with Okadaic acid (O) or calyculin A (●) before the reaction was initiated by the addition of [1-^{14}C]glutamine (1 mM final concentration) and NH_4Cl (1 mM final concentration). Values are nmoles CO_2 produced/mg dry wt cells/30 min and are mean ± SEM; n=3 except at 10^{-5}M (n=2). Control value in the absence of Okadaic acid or calyculin A was 16.5 ± 1.9 nmoles CO_2 produced/mg dry wt cells/30 min. *P<0.05 vs. no added inhibitor.

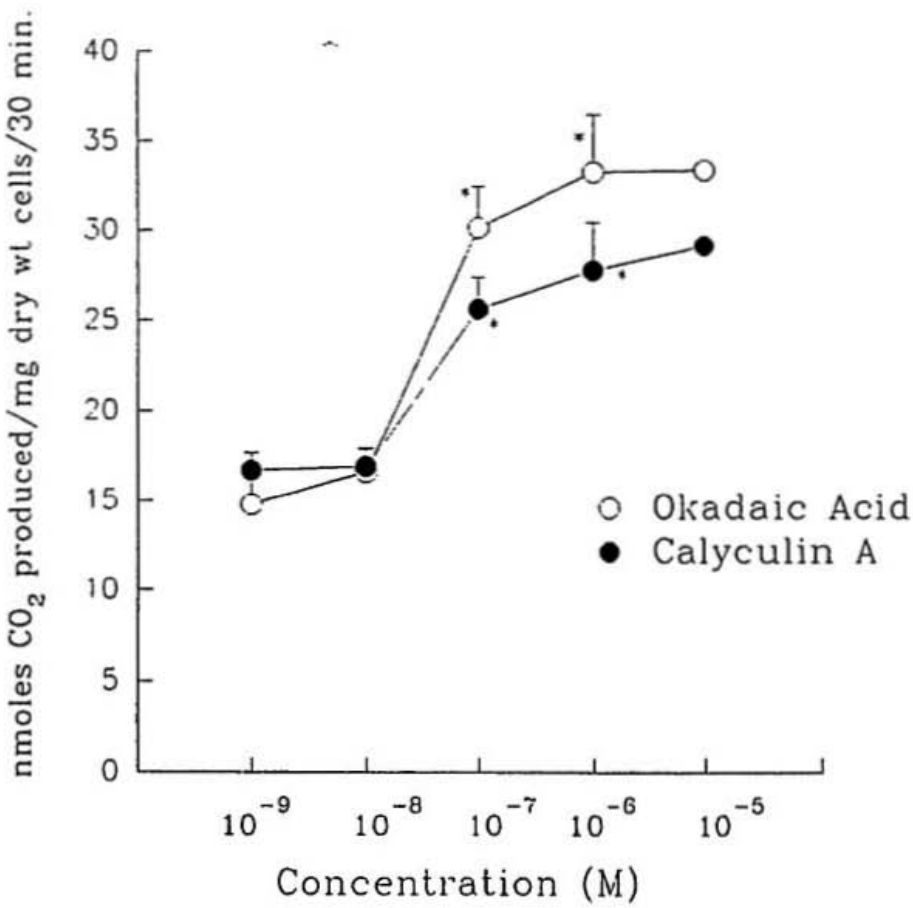
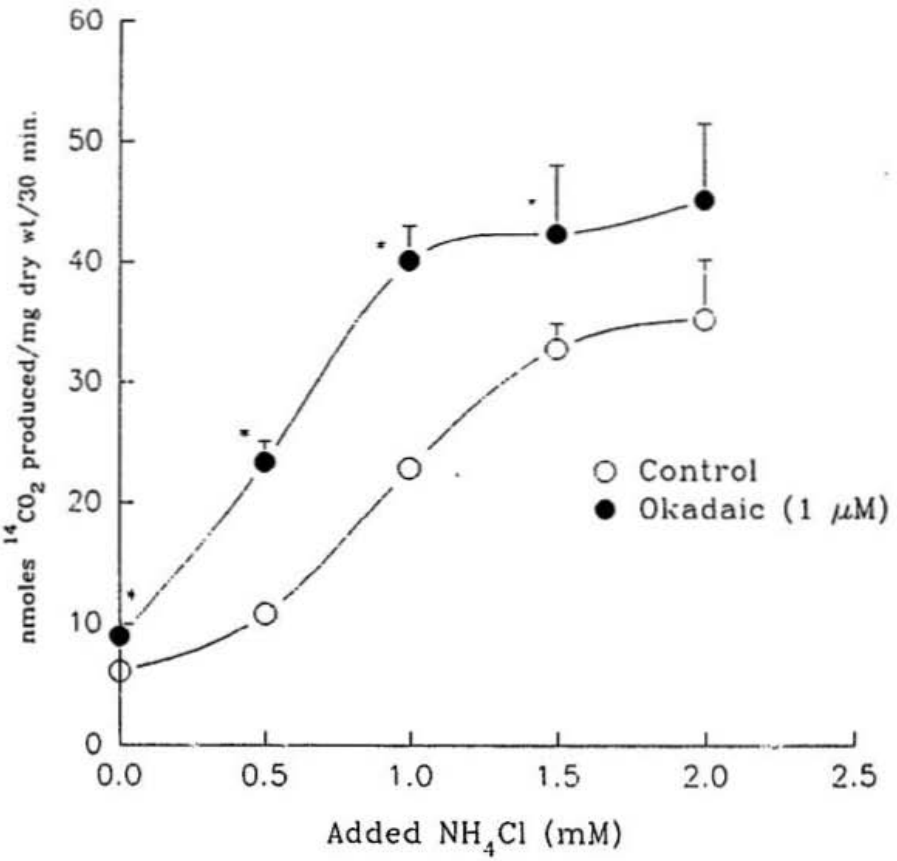

nmoles CO_2 produced/mg dry wt cells/30 min.
40
35
30
25
20
15
10
5
0
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
Concentration (M)
Okadaic Acid
Calyculin A

Figure 4.3. Ammonium Chloride Curve for Flux Through Glutaminase in Isolated Hepatocytes - Effects of 1 μM Okadaic Acid

Okadaic acid (●) was added to the hepatocytes at the start of a 20 minute preincubation at 37°C. The reaction was iniated by the addition of [1-^{14}C]glutamine, 1 mM final concentration, and NH_4Cl. (○) represents control incubations not containing okadaic acid. Values are nmoles CO_2 produced/mg dry wt. cells/30 min. and are mean ± SEM; n=3. * $P<0.05$ vs. control; paired t-test.


60
50
40
30
20
10
0
0.0
0.5
1.0
1.5
2.0
2.5
nmoles $^{14}CO_2$ produced/mg dry wt/30 min.
Added NH_4Cl (mM)
Control
Okadaic (1 μM)

was more evident in the presence of added NH_4Cl. A necessary control experiment was to determine if okadaic acid has a direct effect on glutaminase in isolated mitochondria. The results given in Table 4.1 indicate that it does not. Thus it appears that okadaic acid activates glutaminase through its well defined effects on cytosolic protein phosphatases.

Does glucagon activate the mitochondrial enzyme glutaminase in the same manner as does okadaic acid? To examine this question further, a glucagon dose-response curve was determined in the presence of a submaximal okadaic acid concentration. This concentration, 0.01 μM, produces a slight stimulation of glutaminase flux in isolated hepatocytes. It was thought that this low concentration of okadaic acid might produce a left shift in the glucagon curve. Figure 4.4 shows that this was not the case. This may indicate that glucagon is operating through a separate signalling system from that stimulated by okadaic acid or that there is more than one signalling "system" operating simultaneously. For example, glucagon may act either through increasing the levels of phosphorylated proteins through a cAMP-dependent phosphorylation and/or by increasing the intracellular concentrations of divalent cations such as calcium (Wakelam et al., 1986). It is possible that something in addition to the activity of phosphorylated protein(s) exercises a high degree of control in the signalling cascade.

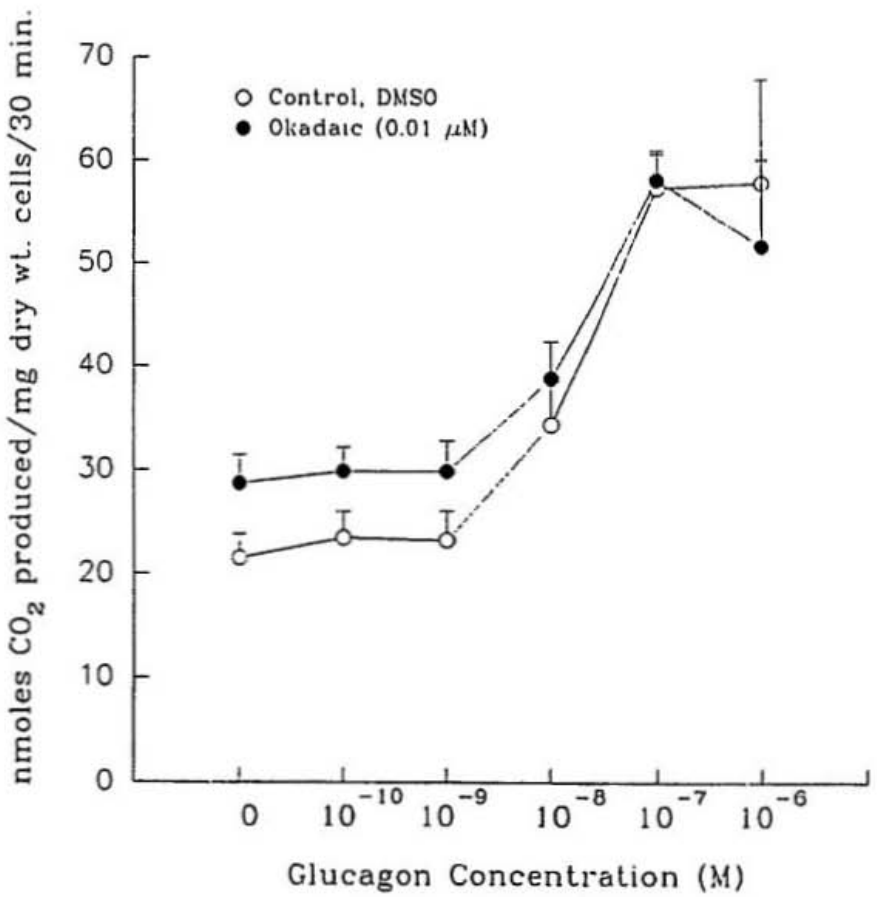
Table 4.1. Glutaminase Flux in Isolated Mitochondria - Effects of Okadaic Acid

Values are mean ± SEM for four separate experiments. Glutaminase flux was determined in the presence of 20 mM glutamine and 10 mM added phosphate. Mitochondria were preincubated at 30°C in the presence of okadaic acid before the addition of glutamine. The amount of glutamate formed was used to determine flux through glutaminase. Glutamate was determined using the glutamate dehydrogenase assay.

	nmoles glutamate formed/mg mitochondrial protein/min.
Control, H_2O	7.9 ± 1.9
Control, DMSO	7.9 ± 1.7
Okadaic Acid ,1 μM	7.6 ± 1.5

Figure 4.4. Glucagon Curve for Glutaminase Flux in Isolated Hepatocytes - Effects of submaximal okadaic acid

Glutaminase flux was determined in isolated hepatocytes using $^{14}CO_2$ release from 1 mM [1-^{14}C]glutamine in the presence of 1 mM NH_4Cl. 0.01 μM okadaic acid was added with the cells and subjected to a 20 minute preincubation at 37°C. Glucagon was added to the flasks, along with the [1-^{14}C]glutamine, after the 20 minute preincubation period. Values are mean ± SEM, n=6.


nmoles CO_2 produced/mg dry wt. cells/30 min.
70
60
50
40
30
20
10
0
Control, DMSO
Okadaic (0.01 μM)
0
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
Glucagon Concentration (M)

Protein kinase A, or cAMP-dependent protein kinase, is a major kinase responsible for phosphorylating cytosolic proteins. My results using the phosphatase inhibitors indicate that phosphorylation of cytosolic protein(s) may be involved in the hormonal signalling process which causes glutaminase flux to be increased. As indicated in **Chapter 1**, Sp-cAMPS is an agonist for protein kinase A. It behaves in a similar fashion to endogenous cAMP by binding to the holoenzyme of protein kinase A allowing the active catalytic subunit to be released. Rp-cAMPS is an antagonist for protein kinase A since when it binds to the holoenzyme of protein kinase A, the active catalytic subunit is not released and thus the kinase is not activated. Both of these cAMP analogues have previously been used in isolated hepatocytes in the examination of the regulation of glycogenolysis and gluconeogenesis (Rothermel et al., 1983; Rothermel et al., 1984). Therefore, I decided to use these compounds to further examine the regulation of glutaminase in isolated hepatocytes. Figure 4.5 shows that Sp-cAMPS stimulates flux through glutaminase in isolated hepatocytes whereas Rp-cAMPS by itself has no effect on glutaminase flux. Figure 4.6 indicates that, like the hormones, Sp-cAMPS only stimulates glutaminase flux in the presence of added ammonium chloride. Again, a necessary control experiment is to examine whether Sp-cAMPS

Figure 4.5. Effects of cAMP analogues, Sp-cAMPS and Rp-cAMPS, on Flux Through Glutaminase in Isolated Hepatocytes

Sp-cAMPS (O) or Rp-cAMPS (●) was added with the hepatocytes and subjected to a 20 minute preincubation at 37°C. After this preincubation [1-^{14}C] glutamine and NH_4Cl were added to give final concentrations of 1 mM and 0.5 mM, respectively. Values are nmoles CO_2 produced/mg dry wt. cells/30 min. and are mean ± SEM; n=3. *P<0.05 vs Rp-cAMPS, paired t-test.

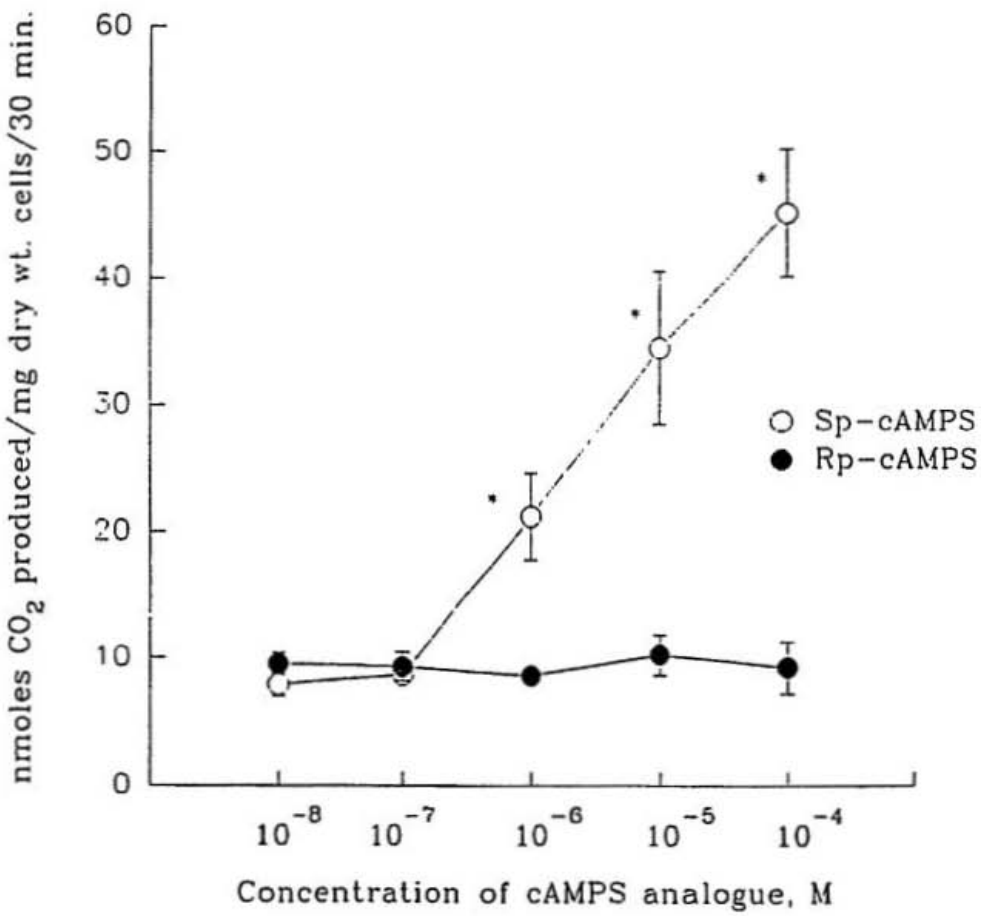

nmoles CO_2 produced/mg dry wt. cells/30 min.
60
50
40
30
20
10
0
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
Concentration of cAMPS analogue, M
Sp-cAMPS
Rp-cAMPS

Figure 4.6. Ammonium Chloride Curve with 10^{-5}M Sp-cAMPS for Flux Through Glutaminase in Isolated Hepatocytes

Sp-cAMPS (●) was added to the hepatocytes at the start of a 20 minute preincubation at 37°C. Control incubations (O) were also preincubated. After preincubation, [1-^{14}]glutamine, 1 mM final concentration, and NH_4Cl was added. Values are nmoles CO_2 produced/mg dry wt. cells/30 min. and are mean ± SEM; n=4. * $P<0.05$ vs. control; paired t-test.

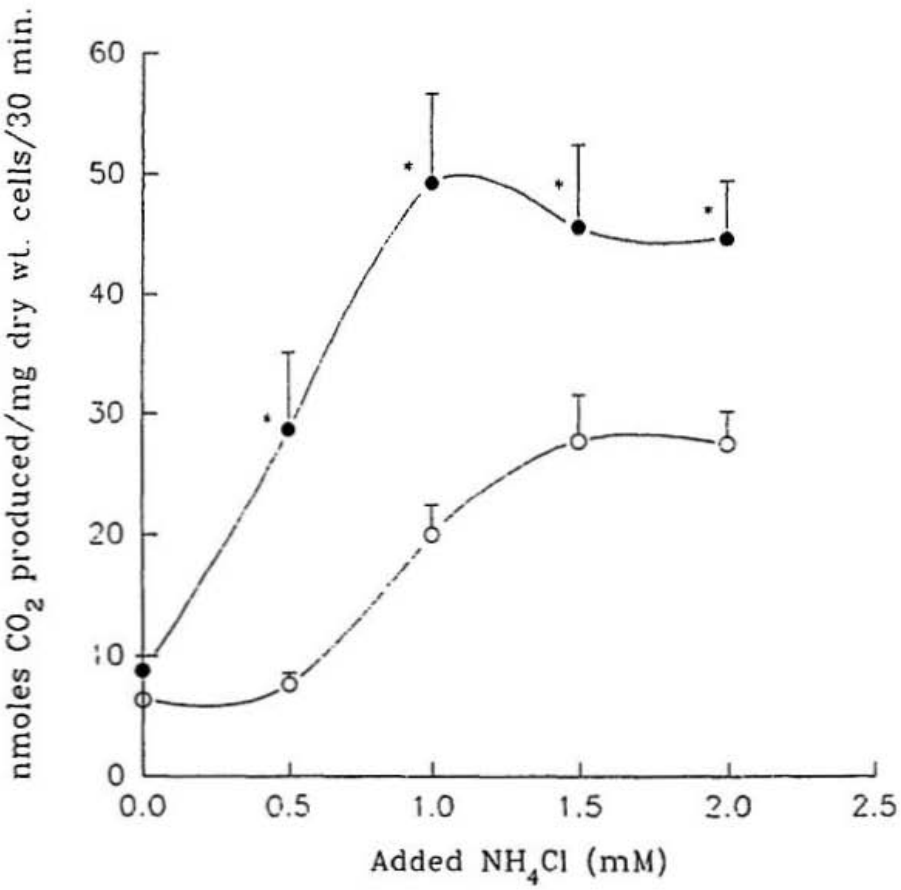

nmoles CO_2 produced/mg dry wt. cells/30 min.
60
50
40
30
20
10
0
0.0
0.5
1.0
1.5
2.0
2.5
Added NH_4Cl (mM)

Table 4.2 Glutaminase flux in isolated mitochondria - effects of Sp-cAMPS

Mitochondria were isolated from the livers of fed rats. The glutaminase assay contained 10 mM Pi and 20 mM glutamine. Values are given as nmoles glutamate formed/mg mitochondrial protein/min. and are mean ± SEM; n=3.

	Glutaminase flux (nmoles/mg protein/min)
Control, H_2O	11.94 ± 3.56
Sp-cAMPS, 10^{-5}M	10.98 ± 2.64

has any effect on glutaminase in isolated intact mitochondria. In this control experiment Sp-cAMPS was found to have no direct effect on glutaminase flux in isolated mitochondria (Table 4.2). These results indicate that Sp-cAMPS stimulates glutaminase by activating a cAMP-dependent protein kinase in the cytosol.

Rp-cAMPS is an antagonist for protein kinase A and has been shown to inhibit the Sp-cAMPS stimulation of glycogenolysis in isolated hepatocytes (Rothermel et al., 1983). The question which arose is whether Rp-cAMPS would also be able to inhibit the Sp-cAMPS stimulated glutaminase flux in isolated hepatocytes. If this were indeed true, it would strengthen the view that Sp-cAMPS, and also cAMP, causes its stimulatory effects on glutaminase by activating a cytosolic cAMP-dependent protein kinase. To determine this it was first necessary to show that the antagonistic effect of Rp-cAMPS was working in our isolated hepatocyte system. As a control, the experiments of Rothermel et al. (1983) were repeated. As is indicated in Figure 4.7, Rp-cAMPS caused a right-shift in the Sp-cAMPS curve for glycogenolysis in our isolated hepatocyte system. Therefore, Rp-cAMPS did antagonize Sp-cAMPS in our system, so we could examine the effect of Rp-cAMPS on Sp-cAMPS stimulted glutaminase flux in isolated hepatocytes. The results given in Figure 4.8 indicate that Rp-cAMPS also antagonizes the stimulation of

Figure 4.7. Effects of Rp-cAMPS on Sp-cAMPS stimulated glycogenolysis in isolated hepatocytes

Glycogenolysis was determined as glucose release from hepatocytes isolated from fed rats. The perfusion media used in the isolation procedure contained 20 mM glucose. 10^{-4} Rp-cAMPS was added with the cells at the start of a 20 minute preincubation before the addition of Sp-cAMPS. After 30 minutes an aliquot was removed from each incubation flask and added to a microcentrifuge tube containing 0.15 ml of 30% perchloric acid (w/v). The supernatant was adjusted to pH 7.0 and this was used for the measurement of glucose by the glucose-6-phosphate dehydrogenase / hexokinase method. Values are mean ± SEM; n=4. *P<0.05 vs control.

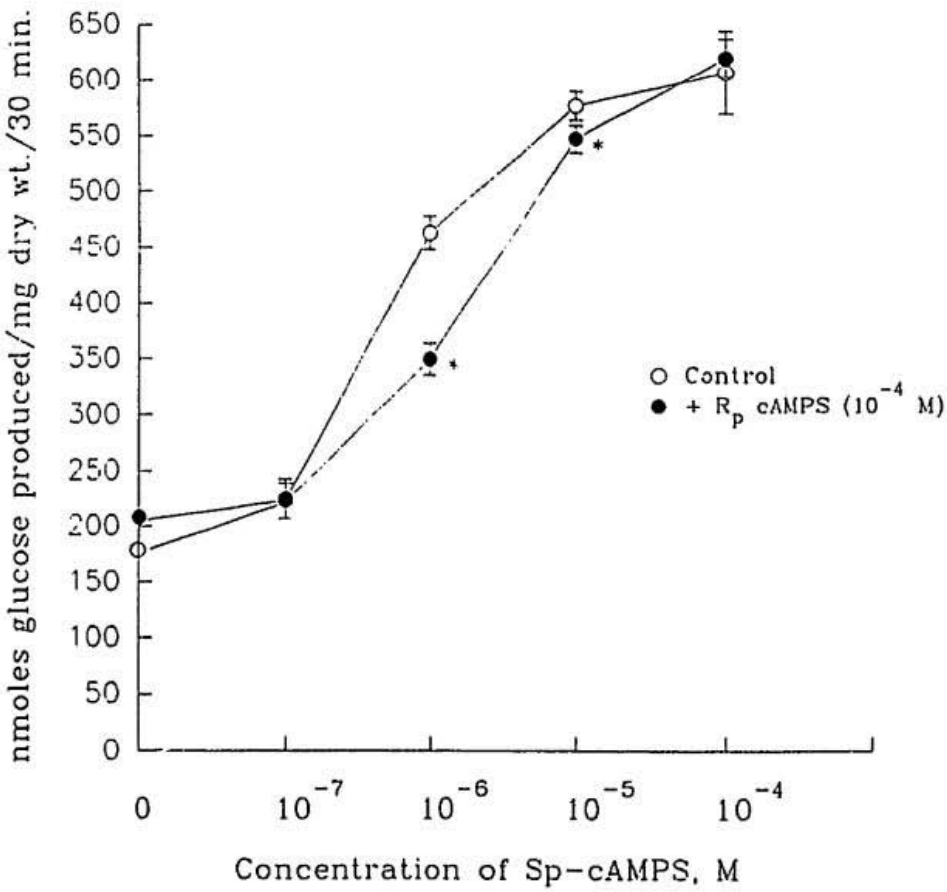
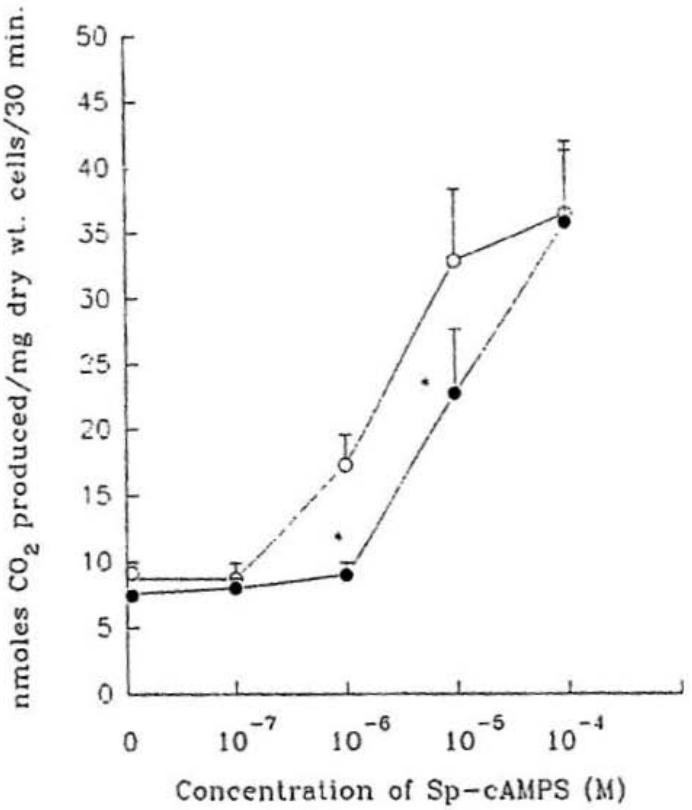

nmoles glucose produced/mg dry wt./30 min.
650
600
550
500
450
400
350
300
250
200
150
100
50
0
0
10^{-7}
10^{-6}
10^{-5}
10^{-4}
Concentration of Sp-cAMPS, M
Control
+ R_p cAMPS (10^{-4} M)
*
*

Figure 4.8. Effects of Rp-cAMPS on Sp-cAMPS stimulated flux through glutaminase in isolated hepatocytes

Glutaminase flux was determined by collecting $^{14}CO_2$ from 1 mM [1-^{14}C]glutamine in the presence of 0.5 mM added NH_4Cl. In (●), hepatocytes were preincubated for 20 minutes with 10^{-4}M Rp-cAMPS before the addition of Sp-cAMPS and [1-^{14}C]glutamine. Control incubations (○) did not contain Rp-cAMPS. Values are mean ± SEM; n=4. *P<0.05 vs. control, paired t-test.


nmoles CO_2 produced/mg dry wt. cells/30 min.
50
45
40
35
30
25
20
15
10
5
0
0
10^{-7}
10^{-6}
10^{-5}
10^{-4}
Concentration of Sp-cAMPS (M)

glutaminase by Sp-cAMPS. This indicates the involvement of cAMP and protein kinase A in the activation of liver glutaminase.

I then determined if Rp-cAMPS, the protein kinase A antagonist, would inhibit the glucagon-induced increase in glutaminase flux in isolated hepatocytes. Rp-cAMPS had been used to inhibit the glucagon-induced increase in glycogenolysis in isolated hepatocytes (Rothermel et al., 1984). Repeating these experiments, I confirmed that Rp-cAMPS caused a right-shift of the glucagon curve for glycogenolysis (Figure 4.9) although the degree of right-shift was less than that reported by Rothermel et al. (1984). This confirms the involvement of protein kinase A in the glucagon-induced increase in glycogenolysis in isolated hepatocytes. No inhibition of glucagon-stimulated glutaminase flux by Rp-cAMPS was observed in isolated hepatocytes (Figure 4.10). This may indicate that glucagon is acting through more than one signalling mechanism or the glucagon concentration required to produce an effect on glutaminase causes such a large increase in endogenous cAMP that Rp-cAMPS is unable to successfully compete for binding to protein kinase A.

Figure 4.9. Effect of Rp-cAMPS on glucagon stimulated glycogenolysis in isolated hepatocytes

Glycogenolysis was determined as glucose production in isolated hepatocytes from fed rats. Rp-cAMPS (10^{-4} M) was preincubated with the hepatocytes before the addition of glucagon. This preincubation was in Krebs-Henseleit medium at 37°C for 20 minutes. Values given are nmoles glucose produced/mg dry wt cells per 30 minutes and are mean ± SEM; n=3. *P<0.05 vs. control; paired t-test.

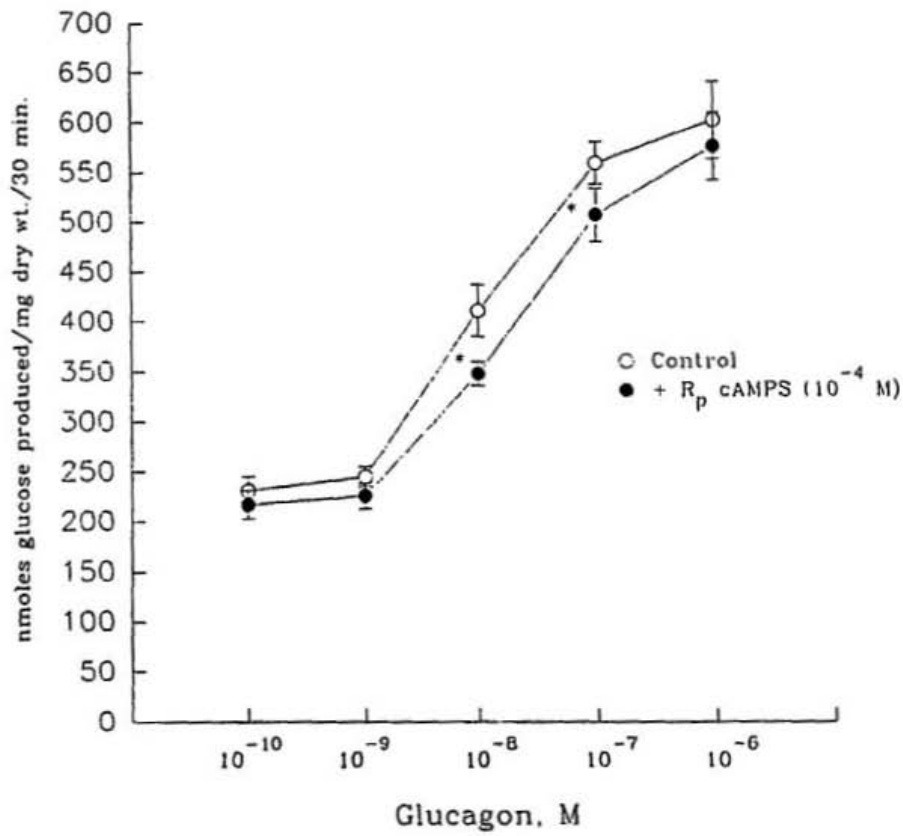
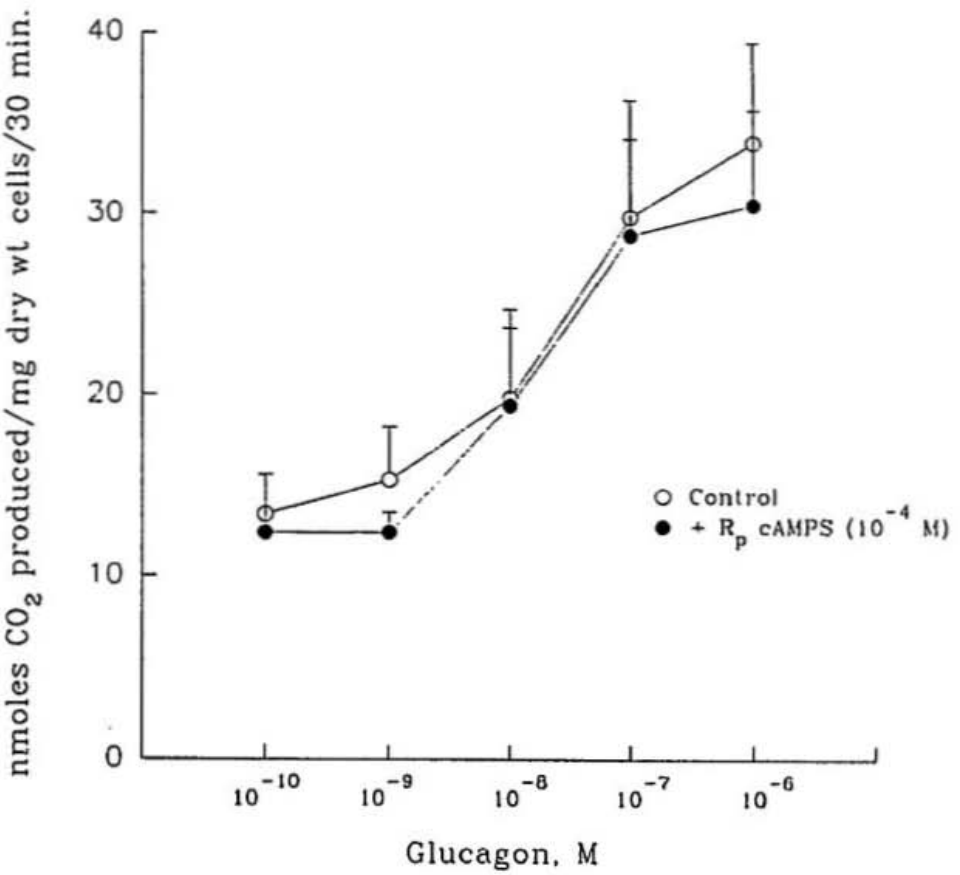

700
650
600
550
500
450
400
350
300
250
200
150
100
50
0
nmoles glucose produced/mg dry wt./30 min.
Control
+ R_p cAMPS (10^{-4} M)
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
Glucagon, M

Figure 4.10. Effects of Rp-cAMPS on glucagon stimulated flux through glutaminase in isolated hepatocytes

Glutaminase flux was determined by collecting $^{14}CO_2$ from 1 mM [1-^{14}C] glutamine in the presence of 1 mM added NH_4Cl. 10^{-4}M Rp-cAMPS was included in the 20 minute preincubation with the hepatocytes before the reaction was initiated by the addition of glucagon, NH_4Cl and [1-^{14}C]glutamine. Values given are nmoles CO_2 produced/mg dry wt. cells per 30 minutes and are mean ± SEM; n=3.


nmoles CO_2 produced/mg dry wt cells/30 min.
40
30
20
10
0
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
Glucagon, M
Control
+ R_p cAMPS (10^{-4} M)

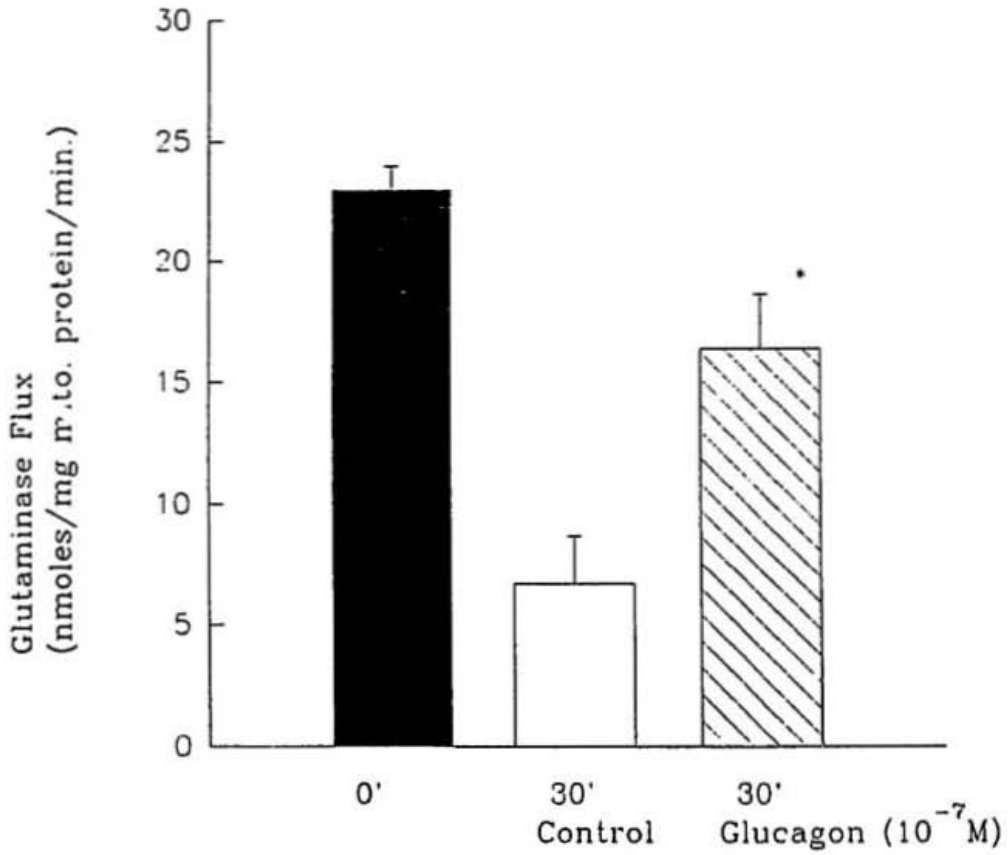
Glutaminase flux in mitochondria isolated from hepatocytes

The stimulation of glutaminase by hormones is characterized by its stability once mitochondria have been isolated. Lacey et al. (1981) have shown that liver mitochondria isolated from glucagon-treated rats exhibit increased flux compared to mitochondria from control rats. This increase was observed in mitochondria isolated 25 minutes after the glucagon injection even though no steps were taken to preserve the phosphorylation state of the proteins. Also in a previous study, Covera and Garcia-Sainz (1983) isolated mitochondria from hepatocytes previously incubated with hormones and determined glutaminase flux in these mitochondria. Their results again indicate that a stable hormonal signal is transmitted into the mitochondria within the intact hepatocyte and this hormonal signal persists when the mitochondria are isolated.

The experiments of Covera and Garcia-Sainz were repeated using glucagon-treated hepatocytes and, as is indicated in Figure 4.11, similar results were obtained. Mitochondria from glucagon-treated hepatocytes have increased glutaminase flux compared to those from control hepatocytes. The so-called 0' mitochondria were isolated from "fresh" hepatocytes not subjected to a 30 minute incubation at 37°C. Interestingly, the glutaminase flux in the 0' mitochondria was higher than in the control mitochondria isolated from hepatocytes which had

Figure 4.11. Glutaminase Flux in Mitochondria Isolated from Hepatocytes - effects of 10^{-7}M Glucagon

Isolated hepatocytes were incubated for 30 minutes at 37°C in the presence of 10^{-7}M glucagon (in 10 mM HCl) or an equivalent amount of 10 mM HCl, control. After 30 minutes, mitochondria were isolated from the hepatocytes using the digitonin method. Mitochondria were also isolated from freshly isolated hepatocytes not exposed to the 30 minute incubation at 37°C. These are represented as 0' incubation. Glutaminase flux was determined in the isolated mitochondria in the presence of 20 mM glutamine and 10 mM phosphate. Values are nmoles glutamate formed/mg mitochondrial protein per minute and are mean ± SEM; n=4. * $P<0.05$ vs. 30 minute control.


Glutaminase Flux
(nmoles/mg m.to. protein/min.)
30
25
20
15
10
5
0
0'
30'
Control
30'
Glucagon (10^{-7}M)
*

been incubated at 37°C for 30 minutes. Covera and Garcia-Sainz did not give the 0'values. It appears that glucagon may "prevent" the decrease in glutaminase flux observed after the 30 minute incubation of the hepatocytes. To examine this further a time course was completed (Figure 4.12).

My previous results show that okadaic acid increases glutaminase flux in isolated hepatocytes. Therefore, the question which arises is whether okadaic acid, like glucagon, results in increased glutaminase flux in mitochondria isolated from treated hepatocytes. The results given in Figure 4.13 indicate that mitochondria from okadaic acid-treated hepatocytes exhibit higher glutaminase flux compared to mitochondria isolated from control hepatocytes. Again as with the glucagon study, the 0' mitochondria exhibited higher glutaminase flux than the 30 minute control. Okadaic acid, like glucagon, prevented this decrease to a large degree. This may indicate the activation of phosphatase(s) during the preincubation. This experiment lends further support for the involvement of cytosolic phosphorylation/dephosphorylation event(s) in the overall process of signal transduction into the mitochondria. Such events may also contribute to the "stable" hormonal effects seen in mitochondria isolated from hormone-treated rats and hepatocytes incubated with hormones.

Figure 4.12. Time course showing the effects of hepatocyte incubation on subsequently isolated mitochondria

Isolated hepatocytes were incubated for the indicated time in the presence (•) or absence (o) of 10^{-7}M glucagon. Mitochondria were isolated from these hepatocytes using the digitonin method. Glutaminase flux was determined in the isolated mitochondria in the presence of 20 mM glutamine and 10 mM phosphate. Values are nmoles glutamate formed/mg mitochondrial protein per minute and are mean ± SEM; n=4. *P<0.05 vs no added glucagon.

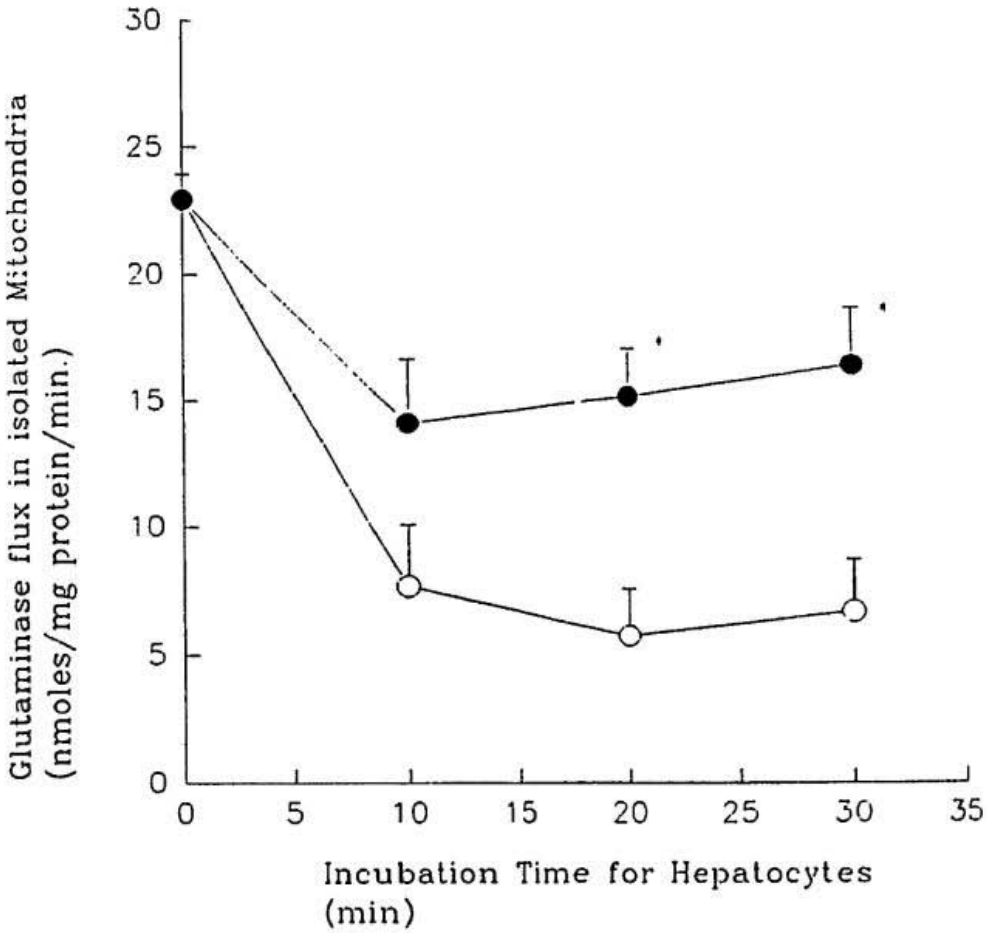
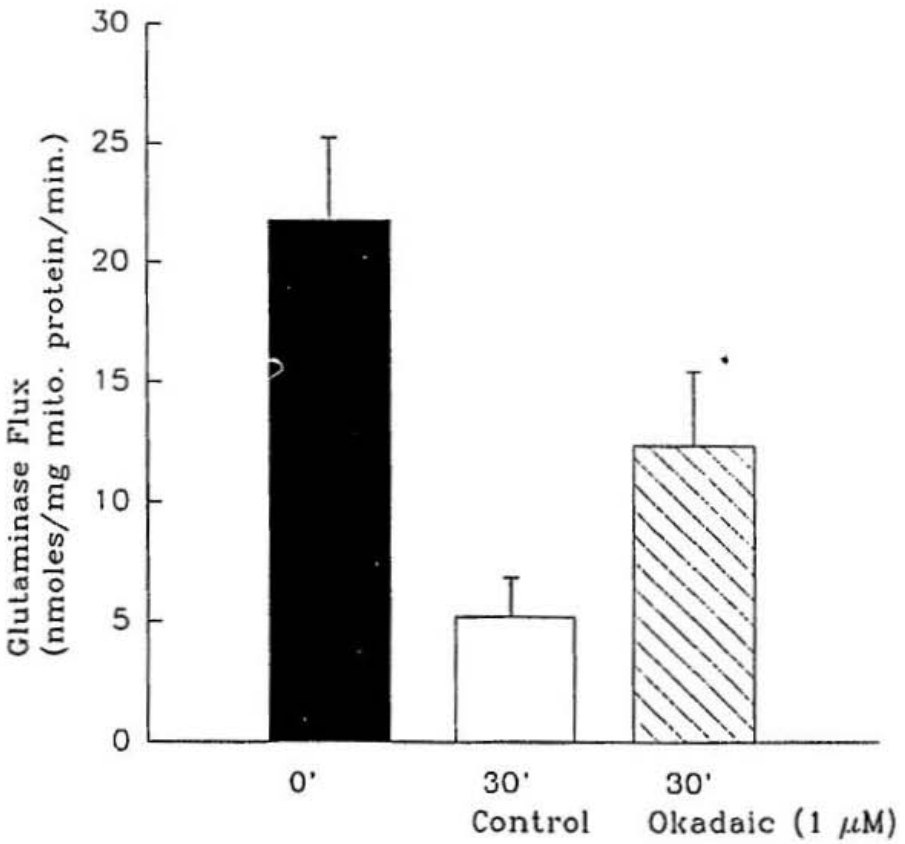

Glutaminase flux in isolated Mitochondria
(nmoles/mg protein/min.)
30
25
20
15
10
5
0
0
5
10
15
20
25
30
35
Incubation Time for Hepatocytes
(min)

Figure 4.13. Glutaminase Flux in Mitochondria Isolated from Hepatocytes - effects of 1 μM Okadaic Acid

Isolated hepatocytes were incubated for 30 minutes at 37°C in the presence of 1 μM okadaic acid (dissolved in DMSO) or an equivalent amount of DMSO, control. After 30 minutes, mitochondria were isolated from the hepatocytes using the digitonin method. Mitochondria were also isolated from freshly isolated hepatocytes not exposed to the 30 minute incubation at 37°C. These are represented as 0' incubation. Glutaminase flux was determined in the isolated mitochondria in the presence of 20 mM glutamine and 10 mM phosphate. Values are nmoles glutamate formed/mg mitochondrial protein per minute and are mean ± SEM; n=7. * $P<0.05$ vs. 30 minute control.


Glutaminase Flux
(nmoles/mg mito. protein/min.)
30
25
20
15
10
5
0
0'
30'
Control
30'
Okadaic (1 μM)

DISCUSSION

Liver glutaminase can be influenced by hormones. This has been previously shown in isolated hepatocytes (Joseph and McGivan, 1978a; Covera and Garcia-Sainz, 1983; Vincent et al., 1989), in perfused liver (Häussinger et al., 1983), and in mitochondria from glucagon-injected rats (Lacey et al., 1981). The results of Figure 4.1 confirm this hormonal stimulation of hepatic glutaminase in isolated hepatocytes. In the absence of added NH_4Cl there was no stimulation of glutaminase by either glucagon or epinephrine. In the presence of added NH_4Cl, the catecholamine epinephrine and the glucogenic hormone glucagon both stimulated $^{14}CO_2$ release. NH_4Cl increased flux through glutaminase and was required for the glucagon effect to be observed. The reason for this is unkown and does not correspond with previous studies. In perfused liver, Häussinger et al. (1983) found a stimulation of glutaminase by glucagon in the absence of added NH_4Cl, and the effects of glucagon and NH_4Cl were additive. In isolated hepatocytes from starved rats, glucagon also stimulated glucose production from glutamine in the absence of added NH_4Cl (Joseph and McGivan, 1978a). Vincent et al. (1989) also found a stimulation of glutaminase by vasopressin in the absence of added NH_4Cl.

The cytosolic protein phosphatase inhibitor okadaic acid also increased flux through glutaminase in isolated

hepatocytes. Okadaic acid's only known effect is the inhibition of protein phosphatase 1 and 2A in the cytosol thereby increasing the level of cytosolic protein phosphorylation (Haystead et al., 1989; Cohen et al., 1990). Okadaic acid did not affect glutaminase flux in isolated intact mitochondria (Table 4.1). This further indicates that the cytosol is required for the actions of okadaic acid to take place. The possibility still exists, however, that okadaic acid directly prevents the dephosphorylation of an outer mitochondrial membrane protein by a cytosolic phosphatase. Haystead et al. (1989) incubated hepatocytes with $^{32}PO_4^{3-}$ and okadaic acid to see if there was an increase in ^{32}P-labelling of proteins. They found a 3 fold increase in phosphorylation levels of proteins in the cytosolic fraction and no increase in the microsomal fraction. In this instance the mitochondrial fraction was not examined which is what is needed to directly determine if okadaic acid causes an increase in the level of mitochondrial protein phosphorylation. Similar studies involving the incubation of hepatocytes with $^{32}PO_4^{3-}$ and glucagon have found no increase in ^{32}P incorporation into mitochondrial membrane proteins (Siess and Wieland, 1979; Halestrap, 1986).

The results shown in Figure 4.2 and Figure 4.3 indicate that increases in protein phosphorylation, presumably extramitochondrial, cause an increase in mitochondrial

glutaminase flux. The results of Figure 4.11 and Figure 4.13 indicate that the hormonal signal persists in mitochondria isolated from hepatocytes previously incubated with glucagon or okadaic acid. Similar results have been reported for other hormones such as adrenaline, vasopressin, and angiotensin II (Covera and Garcia-Sainz, 1983). The finding that okadaic acid produced the same effects as the hormones may indicate that cytosolic phosphorylation/dephosphorylation events play a role in the hormonal signalling in to the mitochondria and may be involved in producing the stable effects seen in the isolated mitochondria.

Protein kinase A, or cAMP-dependent protein kinase, is responsible for the phosphorylation of many cytosolic proteins. Such phosphorylation/dephosphorylation events play important roles in metabolic regulation. The previous results obtained with the protein phosphatase inhibitors strongly suggest that protein phosphorylation is involved in the hormonal signal pathway to the mitochondria. Therefore, it is possible that cAMP-induced phosphorylation may also be involved. To test this idea, the cAMP analogue cAMPS was used in incubations involving isolated hepatocytes. The results of Figure 4.5 and Figure 4.6 clearly show that Sp-cAMPS, a protein kinase A agonist, causes a marked increase in glutaminase flux *in isolated hepatocytes. This Sp-cAMPS* stimulation could be inhibited by the protein kinase A

antagonist, Rp-cAMPS (Figure 4.8). This indicates the involvement of cytosolic cAMP-dependent increases in protein phosphorylation in causing an increase in glutaminase flux within the mitochondria.

The experiments up to this point have shown the hormonal stimulation of glutaminase, and, in addition, have strongly suggested the involvement of increased cytosolic protein phosphorylation caused by the actions of a cAMP-dependent protein kinase in stimulating the mitochondrial-located glutaminase. From these findings it was decided to determine the role of cAMP and cAMP-dependent phosphorylation in the glucagon effect on liver glutaminase. To do this, the protein kinase A antagonist, Rp-cAMPS, was used to determine if a right-shift, or inhibition, of the glucagon curve for glutaminase flux would occur. Such inhibition would strongly indicate that glucagon is also stimulating glutaminase through cytosolic, cAMP-dependent, increases in phosphorylation of proteins. The results of Figure 4.10 indicate that no significant inhibition by Rp-cAMPS occurred.

Glucagon may act through two plasma membrane receptors to produce its effects. This proposal was based on studies involving the glucagon analogue, (1-N-α-trinitrophenylhistidine, 12-homoarginine) glucagon (TH-glucagon). This analogue was found to stimulate glycogenolysis, gluconeogenesis, and urea synthesis in

hepatocytes without activating adenylate cyclase or causing any increase in cAMP. It did, however, stimulate the production of inositol phosphates (Wakelam et al., 1986). From this investigation they proposed the existence of two glucagon receptors. The GR-2 receptor may be linked to adenylate cyclase which results in an increase in cAMP, while the GR-1 receptor may be linked to inositol phospholipid metabolism causing an increase in inositol phosphate and ultimately in Ca^{2+} (Wakelam et al., 1986). In contrast, a recent study has suggested the existence of one receptor which may be responsible for both types of signalling (Jelinek et al., 1993). This group has isolated a cDNA for a rat functional receptor for glucagon by an expression cloning strategy and the receptor protein has been expressed in several kidney cell lines (Jelinek et al., 1993). They found that when the receptor protein is expressed in the kidney cell lines it could still bind glucagon and cause an increase in both cAMP and intracellular calcium. Therefore although these two studies differ with respect to the existence and number of glucagon receptors, they do agree that glucagon causes an increase in both cAMP and Ca^{2+}.

There are several possible explanations for the inability of Rp-cAMPS to inhibit glucagon-stimulated glutaminase flux in isolated hepatocytes. One possibility is that there may be two redundant signalling pathways. Glucagon causes an

increase in both cAMP and Ca^{2+} in the liver. Rp-cAMPS may inhibit the effects of increasing cAMP but the effects of increasing Ca^{2+} may still occur and would result in the glucagon stimulation still being observed. The lack of suitable apparatus for measuring intracellular calcium prevented us from pursuing this further. A second possible explanation is that Rp-cAMPS, which competes with endogenous cAMP for binding to protein kinase A, may not be able to compete with a large increase in cAMP produced by glucagon. In our isolated hepatocyte system, glucagon produces its maximal effects at 10^{-7}M and its half-maximal effect at 10^{-8}M for both glutaminase flux and glycogenolysis. Mine et al. (1990) have also reported a reduced sensitivity of hepatocytes to glucagon action in batch incubation system compared to that which is found in perfused rat liver and perifused hepatocytes. This was attributed to the release of unidentified inhibitor substance(s) by the hepatocytes. This may or may not be what is occuring in our hepatocyte system. What is known, however, is at the effective glucagon concentrations endogenous cAMP levels have been found to increase up to 8-fold in our hepatocyte preparation (Jois et al., 1990). Botelho et al. (1988) state that Rp-cAMPS may not be able to inhibit when glucagon is at a concentration of 10^{-9} M or more due to an increase in endogenous cAMP at the higher glucagon concentrations. Therefore at a concentration of

glucagon necessary to produce an effect on glutaminase in our system the increase in cAMP may be too high for Rp-cAMPS to overcome and this may result in the lack of inhibition observed. Related to this idea is the proposal by Covera et al. (1984) that glucagon causes a large increase in cAMP concentration within certain intracellular compartments whereas the protein kinase A antagonist, Rp-cAMPS, is presumably uniformly distributed throughout the cell. Rp-cAMPS would not be able to overcome the high concentration of endogenous cAMP present in such compartments. Nevertheless this remains speculative.

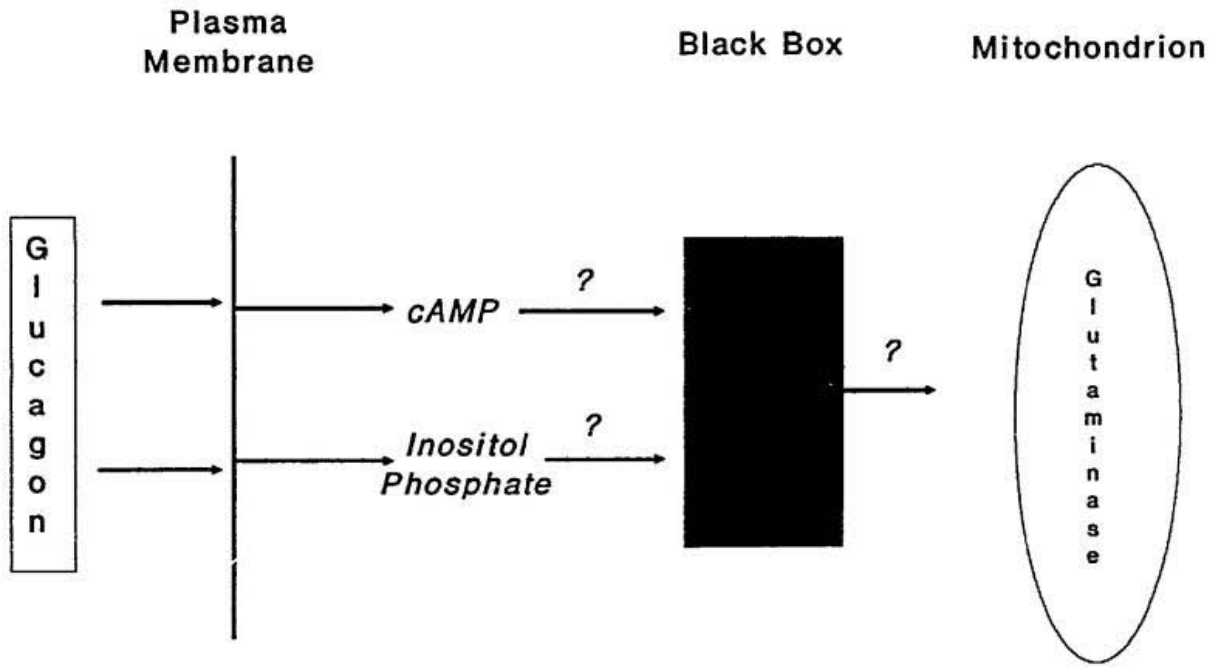
Figure 4.11 and figure 4.13 show that the mitochondria isolated from fresh hepatocytes have higher glutaminase rates than mitochondria from the incubated hepatocytes. It appears that in the 0' mitochondria, glutaminase is somewhat stimulated and over time this stimulation is lost in the control incubations but is maintained in the glucagon and okadaic acid incubations. There may be a cycle of phosphorylation/dephosphorylation operating during the hepatocyte incubations. Such a cycle would include the activation of phosphatase(s). Okadaic acid may prevent the dephosphorylation, and hence inactivation, by inhibiting the phosphatase(s). Glucagon may cause an increase in levels of phosphorylation thereby reducing the decrease in glutaminase flux.

One possible explanation for the hormonal stimulation of glutaminase which has not been examined so far is that hormone treatment of isolated hepatocytes results in an increase in glutaminase protein. Such an increase would cause an increase in glutaminase amount rather than an increase in activity of pre-existing enzyme by the hormones. Smith and Watford (1990) have reported that administration of dibutyryl cAMP to rats results in a 2-fold increase in the abundance of hepatic glutaminase mRNA after 5 hours. Our short term incubation of 30 minutes probably does not provide sufficent time for protein synthesis to occur. To test this, hepatocytes were incubated with glucagon and with or without added cycloheximide (0.2 mM), the eukaryotic protein synthesis inhibitor, and glutaminase flux was determined. The results of this experiment indicate that glucagon still stimulated even in the presence of added cycloheximide (107% stimulation by glucagon in the absence of added cycloheximide vs. 110% in the presence of added cycloheximide).

Unanswered questions

The results of this study clearly demonstrate the involvement of protein phosphorylation and cAMP in the signalling and activation of glutaminase by hormones such as glucagon. As is indicated in Figure 4.14 there are still a number of unanswered questions as represented by the "black box". For example, it is not known where the protein or proteins involved in the phosphorylation are located within the hepatocyte, the identity of the protein(s), and whether a cAMP-dependent protein kinase is directly involved in this phosphorylation. If the increased phosphorylation involved in the signalling is cytosolic, as our results seem to indicate, then how is this increase in cytosolic protein phosphorylation relayed to the mitochondria to affect glutaminase? Our results with Rp-cAMPS and glucagon might suggest that, in addition to cAMP, inositol phosphate may be involved in the signalling process. Exactly how this occurs and how, or if, it is related to increases in protein phosphorylation is still uncertain.

Figure 4.14 Unanswered Questions



Signalling and the Activation of Glutaminase by Glucagon

CHAPTER 5
SUMMARY

SUMMARY

1. Streptozotocin-induced diabetes mellitus results in increased flux through glutaminase in isolated rat hepatocytes. This increase is prevented by maintaining diabetic rats on daily insulin injections.

2. Streptozotocin-induced diabetes mellitus results in increased flux through glutaminase in isolated intact rat liver mitochondria. There is also an increase in glutaminase flux at lower phosphate concentrations suggesting a left-shift in the phosphate curve as occurs in mitochondria from glucagon-injected rats (Lacey et al., 1981). Again, daily insulin treatment of the diabetic rats prevents the increase in glutaminase flux.

3. The increase in glutaminase flux in isolated intact liver mitochondria from streptozotocin-induced diabetics cannot be fully accounted for by the increased glutaminase activity. This, along with the left shift of the phosphate curve, agrees with the idea that the hyperglucagonemia associated with diabetes is exerting an effect on glutaminase.

4. My study confirms that hepatic glutaminase is under acute hormonal regulation. In contrast to previous studies hormones

were found to stimulate glutaminase in isolated hepatocytes only in the presence of added NH_4Cl.

5. Cell-permeable cytosolic protein phosphatase inhibitors cause an increase in glutaminase flux in isolated hepatocytes. This effect was stable, existing in mitochondria isolated from okadaic acid-treated hepatocytes.

6. Sp-cAMPS, a protein kinase A agonist, causes an increase in glutaminase flux in isolated hepatocytes. This increase can be inhibited by the protein kinase A antagonist, Rp-cAMPS. Thus increases in cytosolic protein phosphorylation levels, probably through a cAMP-dependent protein kinase, can cause an increase in glutaminase flux within the mitochondria.

7. In isolated hepatocytes, increases in glutaminase flux caused by the addition of glucagon were not inhibited by the protein kinase A antagonist, Rp-cAMPS. This indicates that the signalling mechanism involved in transmitting the glucagon signal into the mitochondria is more complex and may involve more than just an increase in cAMP and protein phosphorylation levels. More investigation is needed in this area.

REFERENCES

Adam, P.A.J. & Haynes Jr., R.C. (1969) Control of hepatic mitochondrial CO_2 fixation by glucagon, epinephrine, and cortisol. J. Biol. Chem. 244: 6444-6450.

Allan, E.H., Chisholm, A.B. & Titheradge, M.A. (1983) Hormonal stimulation of mitochondrial pyruvate carboxylation of filipin-treated hepatocytes. Biochem J. 212: 417-426.

Almdal, T.P., Holst, J.J., Heindorf, H. & Vilstrup, H. (1992) Glucagon immunoneutralization in diabetic rats normalizes urea synthesis and decreases nitrogen wasting. Diabetes 41: 12-16.

Barber, E.F., Handlogten, M.E., Vida, T.A. & Kilberg, M.S. (1982) Neutral amino acid transport in hepatocytes isolated from streptozotocin-induced diabetic rats. J. Biol. Chem. 257: 14960-14967.

Baverel, G. & Lund, P. (1979) A role for bicarbonate in the regulation of mammalian glutamine metabolism. Biochem. J. 184: 599-606.

Bergmeyer, H.U., Bernt, E., Schmidt, F. & Stork, H. (1974) D-glucose: Determination with hexokinase and glucose-6-phosphate dehydrogenase. In: Methods of Enzymatic Analysis, Vol. 3 (Bergmeyer, H. U. ed.) Academic Press, Inc., N.Y. pp: 1196-1201.

Bernt, E. & Bergmeyer, H.U. (1974) L-glutamate: UV-assay with glutamate dehydrogenase and NAD. In: Methods of Enzymatic Analysis, Vol. 4, (Bergmeyer, H. U. ed.) Academic Press, Inc., N.Y. pp: 1704-1708.

Blackburn, E.H., Hird, F.J.R. & Jones, I.K. (1972) Metabolism of glutamine and ammonia in rat liver: the effects of N-acetylglutamate and phosphate. Arch. Biochem. Biophys. 152: 265-271.

Bode, A.M., Byrd, S. & Klug, G.A. (1990) The relationship between plasma free fatty acids and liver mitochondria function in vivo. Biochim. Biophys. Acta. 1047: 161-167.

Botelho, L.H.P., Rothermel, J.D., Coombs, R.V. & Jastroff, B. (1988) cAMP analog antagonists of cAMP action. Methods in Enzymology 159: 159-172.

Brignone, J.A., Campos De Brignone, C.M., Rodriguez, R.R., Badano, B.N. & Stoppani, A.O.M. (1982) Modified oscillation behavior and decreased D-3-hydroxybutyrate dehydrogenase activity in diabetic rat liver mitochondria. Arch. Biochem. Biophys. 214: 581-588.

Brosnan, J.T., McPhee, P., Hall, B. & Parry, D.M. (1978) Renal glutamine metabolism in rats fed high-protein diets. Am. J. Physiol. 235: E261-265.

Brosnan, J.T., Man, K.C., Colbourne, S.A. & Brosnan, M.E. (1983) Interorgan metabolism of amino acids in streptozotocin-diabetic ketoacidic rat. Am. J. Physiol. 244: E151-158.

Brubaker, P.L., Kazumi, T., Hirano, T., Vranic, M., & Steiner, G. (1991) Failure of chronic hyperinsulinemia to suppress pancreatic glucagon in vivo in the rat. Can. J. Physiol. Pharmacol. 69: 437-443.

Burger, H.J., Gebhardt, R., Mayer, C. & Mecke, D. (1989) Different capacities for amino acid transport in periportal and perivenous hepatocytes isolated by digitonin/collagenase perfusion. Hepatology 9: 22-28.

Charles, R. (1968) Mitochondriale Citrulline Synthese: een Ammoniak Fixerende en ATP Verbruikend Proces. Ph. D. Thesis, University of Amsterdam Amsterdam: Rototype.

Cohen, N.S., Kyan, F.S., Kyan, S.S., Cheung, C.-W. & Raijman, L. (1985) The apparent Km of ammonia for carbamoyl phosphate synthetase (ammonia) in situ. Biochem. J. 229: 205-211.

Cohen, P. & Cohen P.T.W. (1989) Protein phosphatases come of age. J. Biol. Chem. 264: 21435-21348.

Cohen, P., Holmes, C.F.B. & Tsukitani, Y. (1990) Okadaic acid: a new probe for the study of cellular regulation. Trends Biochem. Sci. 15: 98-102.

Connelly, P.A., Botelho, L.H.P., Sisk, R.B. & Garrison, J.C. (1987) A study of the mechanism of glucagon-induced protein phosphorylation in isolated rat hepatocytes using (Sp)-cAMPS and (Rp)-cAMPS, the stimulatory and inhibitory diasteriomers of adenosine 3',5'-phosphorotioate. J. Biol. Chem. 262: 4324-4332.

Covera, S. & Garcia-Sainz, J.A. (1983) Hormonal stimulation of mitochondrial glutaminase: effects of vasopressin, angiotensin II, adrenaline and glucagon. Biochem. J. 210: 957-960.

Covera, S., Huerta-Bahena, J., Peltcn, J.T., Hruby, V.J., Trivedi, D. & Garcia-Sainz, J.A. (1984) Metabolic effects of cyclic AMP levels produced by glucagon, (1-Nα-trinitrophenylhistidine, 12-homoarginine)glucagon and forskolin in isolated rat hepatocytes. Biochim. Biophys. Acta. 804: 434-441.

Covera, S., Jaspers, S. & Pasceri, M. (1991) Acute inhibition of insulin-stimulated glucose transport by the phosphatase inhibitor, okadaic acid. J. Biol. Chem. 266: 9271-9275.

Curthoys, N.P., Kuhlenschmidt, T., Godfrey, S.S. & Weiss, R.F. (1976) Phosphate-dependent glutaminase from rat kidney: cause of increased activity in response to acidosis and identity with glutaminase from other tissues. Arch. Biochem. Biophys. 172: 162-167.

Errera, M. & Greenstein, J.P. (1949) Phosphate-activated glutaminase in kidney and other tissues. J. Biol. Chem. 178: 495-509.

Ewart, H.S. & Brosnan, J.T. (1993) Rapid activation of hepatic glutaminase in rats fed a high protein meal. Biochem. J. 293: 339-344.

Fafournoux, P., Demigne, C., Remesy, C. & Le Cam, A. (1983) Bidirectional transport of glutamine across the cell membrane in rat liver. Biochem. J. 216: 401-408.

Gaasbeck-Janzen, J.W., Lamers, W.H., Moorman, A.F., De Graaf, A., Los, J.A., & Charles, R. (1984) Immunohistochemical localization of carbamoyl-phosphate synthetase (ammonia) in adult rat liver: evidence for a heterogeneous distribution. J. Histochem. Cytochem. 32: 557-564.

Garrison, J.C. & Haynes Jr., R.C. (1975) The hormonal control of gluconeogeneis by regulation of mitochondrial pyruvate in isolated liver cells. J. Biol. Chem. 250: 2769.

Gebhardt, R. & Mecke, D. (1983) Heterogeneous distribution of glutamine synthetase among rat liver

parenchymal cells in situ and in primary culture. EMBO J. 2: 567-570.

Gebhardt, R. & Kleeman, E. (1987) Hormonal regulation of amino acid transport system N in primary cultures of rat hepatocytes. Eur. J. Biochem. 166: 339-344.

Gebhardt, R., Burger, H.J., Heini, H., Schreiber, K.L. & Mecke, D. (1988) Alterations of hepatic enzyme levels and the acinar distribution of glutamine sythetase in response to experimental liver injury in the rat. Hepatology 8: 822-830.

Gornall, A.G., Bardawill, C.J., & David, M.M. (1949) Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. 177: 751-766.

Grinblat, L., Bedetti, C.D. & Stoppani, A.O.M. (1988) Calcium transport and energy coupling in diabetic rat liver mitochondria. Biochemistry International 17: 329-335.

Guha, S.R. (1962) Glutaminases of guinea pig liver mitochondria. Enzymologia, D. Haag 24: 310-326.

Guzman, M. & Castro, J. (1991) Okadaic acid stimulates carnitine palmitoyl transferase I activity and palmitate oxidation in isolated rat hepatocytes. FEBS Lett. 291: 105-108.

Halestrap, A.P. (1986) Hormonal control of liver mitochondrial metabolism. In: Hormonal control of gluconeogenesis, Vol.III. Hormonal effects on mitochondrial and protein phosphorylation (Kraus-Friedmann, N., ed.), CRC Press Boca Raton, FL, pp: 31-48.

Halestrap, A.P. (1989) The regulation of the matrix volume of mammalian mitochondria in vivo and in vitro and its role in the control of mitochondrial metabolism. Biochim. Biophys. Acta 973: 355-382.

Halestrap, A.P., Davidson, A.M. & Potter, W.D. (1990) Mechanisms involved in the hormonal regulation of mitochondrial function through changes in the matrix volume. Biochim. Biophys. Acta 1018: 278-281.

Harano, Y., DePalma, R.G., Lavine, L. & Miller, M. (1972) Fatty acid oxidation, oxidative phosphorylation and ultrastructure of mitochondria in the diabetic rat liver. Diabetes 21: 257-269.

Hardie, D.G., Haystead, T.A. & Sim, A.T.R. (1991) Use of okadaic acid to inhibit protein phosphatases in intact cells. Methods in Enzymology 201: 469-471.

Häussinger, D. (1983) Hepatocyte heterogeneity in glutamine and ammonia metabolism and the role of an intracellular glutamine cycle during ureogenesis in perfused rat liver. Eur. J. Biochem. 133: 269-275.

Häussinger, D. (1990) Nitrogen metabolism in liver: structural and functional organisation and physiological relevance. Biochem. J. 267: 281-290.

Häussinger, D., Akerboom, T.P.M. & Sies, H. (1980) The role of pH and the lack of a requirement for hydrogencarbonate in the regulation of hepatic glutamine metabolism. Hoppe-Seyler's Z. Physiol. Chem. 361: 995-1001.

Häussinger, D., Gerok, W. & Sies, H. (1983) Regulation of flux through glutaminase and glutamine synthetase in isolated perfused rat liver. Biochim. Biophys. Acta 755: 272-278.

Häussinger, D., Soboll, S., Meijer, A.J., Gerok, W., Tager, J.M. & Sies, H. (1985) Role of plasma membrane

transport in hepatic glutamine metabolism. Eur. J. Biochem. 152: 597-603.

Haystead, T.A.J., Sim, A.T.R., Carling, D., Honnor, R.C., Tsukitani, Y., Cohen, P. & Hardie, D.G. (1989) Effects of the tumor promoter okadaic acid on intracellular protein phosphorylation and metabolism. Nature 337: 78-81.

Hengsens, H.E.S.J., Verhoeven, A.J. & Meijer, A.J. (1980) The relationship between intramitochondrial N-acetylglutamate and activity of carbamoyl-phosphate synthetase (ammonia). Eur. J. Biochem. 107: 197-205.

Ishihara, H., Martin, B.L., Brautigan, D.L., Karaki, H., Ozaki, H., Kato, Y., Fusetani, N., Watabe, S., Hashimito, K., Uemura, D. & Hartshorne, D.J. (1989) Calyculin A and okadaic acid: inhibitors of protein phosphatase activity. Biochem. Biophys. Res. Commun. 159: 871-877.

Jelinek, L.J., Lok, S., Rosenberg, G.B., Smith, R.A., Grant, F.J., Biggs, S., Bensch, P.A., Kuijper, J.L., Sheppard, P.O., Sprecher, C.A., O'Hara, P.J., Foster, D., Walker, K.M., Chen, L.H.J., McKernan, P.A. & Kindsvogel, W. (1993) Expression cloning and signalling properties of the rat glucagon receptor. Science 259: 1614-1616.

Jensen, C.B., Sistare, F.D., Hamman, H.C. & Haynes Jr., R.C. (1983) Stimulation of mitochondrial functions by glucagon treatment: evidence that these effects are not artifacts of mitochondrial isolation. Biochem. J. 210: 819-827.

Jois, M., Hall, B., Fewer, K., & Brosnan, J.T. (1989) Regulation of hepatic glycine catabolism by glucagon. J. Biol. Chem. 264 (6): 3347-3351.

Jois, M., Hall, B., Collett, V.M., & Brosnan, J.T. (1990) Flux through the glycine cleavage system in isolated hepatocytes: effects of glucagon, cAMP, and calcium. Biochem. Cell. Biol. 68: 543-546.

Jois, M., Ewart, H.S. & Brosnan, J.T. (1992) Regulation of glycine catabolism in rat liver mitochondria. Biochem. J. 283: 435-439.

Joseph, S.K. & Meijer, A.J. (1981) The inhibitory effects of sulphydryl reagents on the transport and hydrolysis of glutamine in rat-liver mitochondria. Eur. J. Biochem. 119: 323-329.

Joseph, S.K., Bradford, N.M. & McGivan, J.D. (1978) Characteristics of the transport of alanine, serine, and

glutamine across the plasma membrane of rat liver cells. Biochem. J. 176: 827-836.

Joseph, S.K. & McGivan, J.D. (1978a) The effect of ammonium chloride and glucagon on the metabolism of glutamine in isolated liver cells from starved rats. Biochim. Biophys. Acta 543: 16-28.

Joseph, S.K. & McGivan, J.D. (1978b) The effect of ammonium chloride and bicarbonate on the activity of glutaminase in isolated liver mitochondria. Biochem. J. 176: 837-844.

Joseph, S.K., McGivan, J.D. & Meijer, A.J. (1981) The stimulation of glutamine hydrolysis in isolated rat liver mitochondria by Mg^{2+} depletion and hypo-osmotic incubation conditions. Biochem. J. 194: 35-41.

Kalra, J. & Brosnan, J.T. (1973) Localization of glutaminase in rat liver. FEBS Lett. 37: 325-328.

Kilberg, M.S., Handlogten, M.E., & Christensen, H.N. (1980) Characteristics of an amino acid transport system in rat liver for glutamine, asparagine, histidine, and closely related analogs. J. Biol. Chem. 255: 4011-4019.

Klingman, J.D. & Handler, P. (1958) Partial purification and properties of renal glutaminase. J. Biol. Chem. 232: 369-380.

Klug, G.A., Krause, J., Ostlund, A.K., Knoll, G. & Brdiczka, D. (1984) Alterations in liver mitochondrial function as a result of fasting and exhaustive exercise. Biochim. Biophys. Acta. 764: 272-282.

Kovacevic, Z. & McGivan, J.D. (1983) Mitochondrial metabolism of glutamine and glutamate and its physiological significance. Physiol. Rev. 63: 547-605.

Krebs, H.A. (1935) Metabolism of amino acids IV. the synthesis of glutamine from glutamic acid and ammonia and the enzymic hydrolysis of glutamine in animal tissues. Biochem. J. 29: 1951-1969.

Krebs, H.A., Cornell, N.W., Lund, P. & Hems, R. (1974) Isolated liver cells as experimental material. In: Regulation of hepatic metabolism. Munksgaard, Copenhagen: 726-750.

Kvamme, E., Tveit, B. & Svenneby, G. (1970) Glutaminase from pig renal cortex I. purification and general properties. J. Biol. Chem. 245: 1871-1877.

Kvamme, E., Torgner, I.A. & Roberg, B. (1991) Evidence indicating that pig renal phosphate-activated glutaminase has a predominantly external localization in the inner mitochondrial membrane. J. Biol. Chem 266: 13185-13192.

Lacey, J.H., Bradford, N.M., Joseph, S.K. & McGivan, J.D. (1981) Increased activity of phosphate-dependent glutaminase in liver mitochondria as a result of glucagon treatment of rats. Biochem. J. 194: 29-33.

Lautt, W.W. & Greenway, C.V. (1987) Conceptual overview of the hepatic vascular bed. Hepatology 7: 952-963.

Lawerence, J.C., Hiken, J.F. & James, D.E. (1990) Stimulation of glucose transport and glucose transporter phosphorylation by okadaic acid in rat adipocytes. J. Biol. Chem. 265: 19768-19775.

Lenzen, C., Soboll, S., & Haussinger, D. (1987) pH control of hepatic glutamine degradation: role of transport. Eur. J. Biochem. 166: 483-488.

Lerner, E., Shug, A.L., Elson, C. & Shrago, E. (1972) Reversible inhibition of adenine nucleotide translocation by long chain coenzyme A esters in liver mitochondria of diabetic and hibernating animals. J. Biol. Chem. 247: 1513-1519.

Low, S., Salter, M., Knowles, R.G., Rennie, M.J. & Pogson, C.I. (1990) Effect of L-glutamate-gamma-hydrazide on the transport and metabolism in rat liver cells and isolated mitochondria. Biochem. Soc. Trans. 18: 1239-1240.

Low, S.Y., Taylor, P.M., Ahmed, A., Pogson, C.I. & Rennie, M.J. (1991) Substrate-specifity of glutaminase transporters in membrane vesicles from rat liver and skeletal muscle investigated using amino acid analogues. Biochem. J. 278: 105-111.

Low, S.Y., Taylor, P.M., Hundal, H.S., Pogson, C.I. & Rennie, M.J. (1992) Transport of L-glutamine and L-glutamate across sinusoidal membranes of rat liver. Biochem. J. 284: 333-340.

Lund, P. (1974) L-glutamine: Determination with glutaminase and glutamate dehydrogenase. In: Methods of Enzymatic Analysis, Vol. 4 (Bergmeyer, H, U, ed.) Academic Press, Inc. N.Y. pp: 1719-1722.

McGivan, J.D. & Bradford, N.M. (1983) Characteristics of the activation of glutaminase by ammonia in sonicated rat-liver mitochondria. Biochim. Biophys. Acta 759: 296-302.

McGivan, J.D., Lacey, J.H. & Joseph, S.K. (1980) Localisation and some properties of phosphate-dependent glutaminase in disrupted liver mitochondria. Biochem. J. 192: 537-542.

McGivan, J.D., Vadner, M., Lacey, J. & Bradford, N. (1985) Rat liver glutaminase: regulation by reversible interaction with the mitochondrial membrane. Eur. J. Biochem. 148: 323-327.

Meijer, A.J. & Verhoeven, A.J. (1986) Regulation of hepatic glutamine metabolism. Biochem. Soc. Trans. 14: 1001-1004.

Meister, A. (1968) The specificity of glutamine synthetase and its relationship to substrate conformation at the active site. Adv. Enzymol. 31: 183-218.

Mine, T., Kojima, I. & Ogata, E. (1990) Difference in sensitivity to glucagon action in three different rat liver systems. Metabolism 39: 321-326.

Patel, D.G. (1983) Effect of prolonged insulin treatment on blunted plasma catecholamine and glucagon increase during insulin hypoglycemia in streptozotocin diabetic rats. Metabolism 32: 377-382.

Patel, M. & McGivan, J.D. (1984) Partial purification and properties of rat liver glutaminase. Biochem. J. 220: 583-590.

Pinkus, L.M. & Windmueller, H.G. (1977) Phosphate-dependent glutaminase of small intestine: localization and role in intestinal metabolism. Arch. Biochem. Biophys. 182: 506-517.

Pogson, C.I., Low, S.Y., Knowles, R.G., Salter, M., & Rennie, M.J. (1990) Application of metabolic control theory to amino acid metabolism in liver. In: Regulation of Hepatic

Function (Quistoff, B; Grunnett, N; Ness-Thysen, J, eds) Alfred Benzen Symposium 30: 262-272.

Rothermel, J. D., Stec, W.J., Baraniak, J., Jastroff, B. & Botelho, L.H.P. (1983) Inhibition of glycogenolysis in isolated rat hepatocytes by the Rp diastereomer of adenosine cyclic 3',5'-phosphorothioate. J. Biol. Chem. 258: 12125-12128.

Rothermel, J.D., Jastroff, B. & Botelho, L.H.P. (1984) Inhibition of glucagon-induced glycogenolysis in isolated rat hepatocytes by the Rp diastereomer of adenosine cyclic 3',5'-phosphorothioate. J. Biol. Chem. 259: 8151-8155.

Sayre, F.W. & Roberts, E. (1958) Preparation and some properties of a phosphate-activated glutaminase from kidney. J. Biol. Chem. 233: 1128-1134.

Schrock, H. & Goldstein, L. (1981) Interorgan relationships for glutamine metabolism in normal and acidotic rats. Am. J. Physiol. 240: E519-E525.

Siess, E.A. & Wieland, O.H. (1979) Isolated hepatocytes as a model for the study of stable glucagon effects on mitochondrial respiratory functions. FEBS Lett. 101: 277-281.

Siess, E.A., Fahimi, F.M. & Wieland, O.H. (1981) Evidence that glucagon stabilizes rather than activates mitochondrial functions in rat liver. Hoppe-Seyler's Z. Physiol. Chem. 362: 1643-1651.

Smith, E.M. & Watford, M. (1988) Rat hepatic glutaminase: purification and immunochemical characterisation. Arch. Biochem. Biophys. 260: 740-751.

Smith, E.M. & Watford, M. (1990) Molecular cloning of a cDNA for rat hepatic glutaminase: sequence similarity to kidney-type glutaminase. J. Biol. Chem. 265: 10631-10636.

Snodgrass, P.J. & Lund, P. (1984) Allosteric properties of phosphate-activated glutaminase of human liver mitochondria. Biochim. Biophys. Acta 798: 21-27.

Soboll, S. & Stucki, J. (1985) Regulation of the degree of coupling of oxidative phosphorylation in intact rat liver. Biochim. Biophys. Acta. 1047: 161-167.

Soboll, S., Lenzen, C., Rettich, D., Grundel, S. & Ziegler, B. (1991) Characterisation of glutamine uptake in rat liver mitochondria. Eur. J. Biochem. 197: 113-117.

Suganuma, M., Fujiki, H., Furuya-Suguri, H., Yoshizawa, S., Yasumoto, S., Kato, Y., Fusetani, N. & Sugimura, T. (1990) Calyculin A, an inhibitor of protein phosphatases, a potent tumour promoter on CD-1 mouse skin. Cancer Research 50: 3521-3525.

Svenneby, G., Torgner, I.A. & Kvamme, E. (1973) Purification of phosphate-dependent pig brain glutaminase. J. Neurochem. 20: 1217-1224.

Szweda, L.I. & Atkinson, D.E. (1989) Response of rat liver glutaminase to pH: mediation by phosphate and ammonium ions. J. Biol. Chem. 264: 15357-15360.

Szweda, L.I. & Atkinson, D.E. (1990a) Response of rat liver glutaminase to magnesium ion. Biochim. Biophys. Acta 1041: 201-206.

Szweda,L.I. & Atkinson,D.E. (1990b) Response of rat liver glutaminase to pH, ammonium, and citrate: possible regulatory role of glutaminase in ureagenesis. J. Biol. Chem. 265(34): 20869-20873.

Tachibana, Y., Scheuer, P.J., Tsukitani, Y., Kikuchi, H., Van Engen, D., Clardy, J., Giopichard, Y. & Schmitz, F. J. (1981) Okadaic acid, a cytotoxic polyether from two marine sponges of the genus Halichondria. J. Am. Chem. Soc. 103: 2469-2471.

Taki, A., Bialojan, C., Troschka, M. & Ruegg, J.C. (1987) Smooth muscle myosin phosphatase inhibition and force enhancement by black sponge toxin. FEBS Lett. 217: 81-84.

Tamarappoo, B.K., Handlogten, M.E., Laine, R.O., Serrano, M.A., Dugan, J. & Kilberg, M.S. (1992) Identification of the protein responsible for hepatic system N amino acid transport activity. J. Biol. Chem. 267(4): 2370-2374.

Unger, R.H. & Orci, L. (1981) Glucagon and the A cell: physiology and pathophysiology. New Engl. J. Med. 304: 1575-1580.

Van Haastert, P.J.M. & Kien, E. (1983) Binding of cAMP derivatives to dictyostelium discoideum cells. Activation mechanism of the cell surface receptor. J. Biol. Chem. 258: 9636-9642.

Vargas, A.M., Halestrap, A.P. & Denton, R.M. (1982) The effects of glucagon, phenylephrine and insulin on the phosphorylation of cytoplasmic, mitochondrial and membrane-bound proteins of intact liver cells from starved rats. Biochem. J. 208: 221-229.

Verhoeven, A.J., Van Iwaarden, J.F., Joseph, S.K. & Meijer, A.J. (1983) Control of rat-liver glutaminase by ammonia and pH. Eur. J. Biochem. 133: 241-244.

Verhoeven, A.J., Estrela, J.M. & Meijer, A.J. (1985) alpha-adrenergic stimulation of glutamine metabolism in isolated rat hepatocytes. Biochem. J. 230: 457-463.

Vincent, N., Martin, G. & Baverel, G. (1989) Simultaneous synthesis and degradation of glutamine in isolated rat liver cells. Effect of vasopressin. Biochim. Biophys. Acta 1014: 184-188.

Wakelam, M.J.O., Murphy, G.J., Hruby, V.J. & Houslay, M.D. (1986) Activation of two signal-transduction systems in hepatocytes by glucagon. Nature 323: 68-71.

Watford, M. & Smith, E.M. (1990) Distribution of hepatic glutaminase activity and mRNA in perivenous and periportal hepatocytes. Biochem. J. 267: 265-267.

Watford, M., Smith, E.M. & Erbelding, E.J. (1984) The regulation of phosphate-activated glutaminase and glutamine metabolism in the streptozotocin diabetic rat. Biochem. J. 224: 207-221.

Wingrove, D.E., Amatruda, J.M., & Gunter, T.E. (1984) Glucagon effects on the membrane potential and calcium uptake rate of rat liver mitochondria. J. Biol. Chem. 245: 375-379.

Yamazaki, R.K. & Graetz, G.S. (1977) Glucagon stimulation of citrulline formation in isolated hepatic mitochondria. Arch. Biochem. Biophys. 178: 19-23.