

HORMONAL REGULATION OF GLYCINE CLEAVAGE
ENZYME SYSTEM

CENTRE FOR NEWFOUNDLAND STUDIES

**TOTAL OF 10 PAGES ONLY
MAY BE XEROXED**

(Without Author's Permission)

GEHAN M.Y. MABROUK



HORMONAL REGULATION OF GLYCINE CLEAVAGE ENZYME SYSTEM

by

Gehan M.Y. Mabrouk, B.V.Sc.

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

Department of Biochemistry
Memorial University of Newfoundland

May, 1996

St. John's

Newfoundland



National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395, Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file: Votre référence

Our file: Notre référence

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-612-17622-3

Canada

Abstract

The glycine cleavage enzyme complex is a mitochondrial enzyme that is known to be activated by hormones such as glucagon. The effects of several glucagon-related peptides such as glucagon-like peptide-1 (7-36) amide, oxyntomodulin and glicentin, as well as miniglucagon, on the glycine cleavage system were examined in isolated rat hepatocytes. Oxyntomodulin and glicentin were found to stimulate the glycine cleavage system flux through their interaction with the glucagon receptor. Glucagon-like peptide-1 and miniglucagon had no effect on the glycine cleavage system.

Although the stimulation of the glycine cleavage system by glucagon has been demonstrated previously, it is not known how the hormonal signal is transmitted to the mitochondria. This question is examined in this thesis. The cell-permeable protein phosphatase inhibitor, okadaic acid, was found to stimulate the flux through the glycine cleavage system. The protein kinase A agonist, Sp-cAMPS was also found to stimulate the glycine cleavage system flux, an effect that was inhibited by the protein kinase A antagonist, Rp-8-Br-cAMPS. These results suggest that protein kinase A dependent phosphorylation of cytosolic protein(s) can affect the glycine cleavage system. The role of intracellular calcium in the

regulation of the glycine cleavage system was examined using the calcium-mobilizing hormones, vasopressin and angiotensin II, both of which stimulate the glycine cleavage system. Finally, the role of protein kinase C in the regulation of glycine cleavage system was examined using the phorbol ester, Phorbol 12-myristate 13-acetate (PMA). PMA had no effect on the glycine cleavage system or on the glucagon-stimulated glycine cleavage system flux, which suggests no role for protein kinase C in the activation of the glycine cleavage system.

ACKNOWLEDEMENT

I wish to express my deepest gratitude to my supervisor, Dr. J.T. Brosnan for his guidance, support and patience. I wish to acknowledge the support and guidance of my supervisory committee, Dr. M.E. Brosnan and Dr. P. Davis.

Special thanks are given to the following people for their helpfulness and support throughout my programme: Mrs. Beatrice Hall, Mr. Craig Skinner, Daniel O'Sullivan, Stephen Squires, Kimberly Purchase, Dr. James House, Dr. Stephen Ewart and Dalong Qian. I would like also to thank all of the members of the Biochemistry Department at Memorial for their support and helpfulness.

I wish to acknowledge the Canadian Diabetes Association and the Medical Research Council of Canada for funding my research.

In conclusion, I wish to dedicate this thesis to my husband and my daughter for their support and patience. Thank you both very much, without you this thesis may have never been completed. I wish also to dedicate this thesis to my parents. Thank you for your support and understanding.

TABLE OF CONTENTS

Abstract.....	ii
Acknowledgments.....	iv
List of figures.....	viii
List of tables.....	x
List of abbreviations.....	xi
 Chapter 1 Introduction	
Introduction.....	2
Routes of glycine catabolism	
D-amino acid oxidase pathway.....	4
Glycine cleavage enzyme system.....	5
Glycine cleavage system in different	
organisms.....	6
Glycine cleavage system in rat.....	6
GCS location and components.....	7
T-protein.....	7
L-protein.....	8
P-protein.....	8
H-protein.....	8
Mechanism of action.....	9
Regulation of GCS.....	10
Branched chain α -keto acid.....	10
Oxidation-reduction in the mitochondrial	
pyridine nucleotides.....	12
Dietary protein.....	13
Hormonal regulation.....	14
Defects in glycine catabolism.....	17
Glucagon-related peptides.....	19
Oxyntomodulin.....	20
GLP-1.....	23
Glicentin.....	25
Miniglucagon.....	26
Glucagon receptor antagonists.....	28
Des-His ¹ -Glu ⁹ -glucagon amide and its	
biological activities.....	29
Des-His ¹ -Nle ⁹ -Ala ¹¹ Ala ¹⁶ -glucagon amide.....	30
Okadaic acid.....	32
Okadaic acid as a probe for identifying	
biological processes that are controlled	
by phosphorylation.....	35
Specificity of okadaic acid.....	35
Cyclic AMP analogues.....	36
Specificity of Rp-cAMPS.....	39
Calcium-mobilizing hormones.....	39

Role of protein kinase C.....	43
Problems of investigation.....	44
Chapter 2 Materials and methods.....	47
Materials.....	48
Chemicals.....	48
Animals.....	48
Methods.....	49
Preparation of hepatocytes.....	49
Determination of cell quantity.....	50
Determination of cell viability.....	50
Preparation of mitochondria.....	51
Measurement of flux through GCS in isolated hepatocytes.....	52
GCS flux in isolated mitochondria.....	53
Viability of using $^{14}\text{CO}_2$ release from [1- ^{14}C]glycine to determine the flux through GCS.....	53
Glycogenolysis in isolated hepatocytes....	55
Attempts to increase the sensitivity of the hepatocytes.....	56
Removal of damaged hepatocytes with Percoll	56
Reduction of the duration of incubation....	58
Reduction of the concentration of the hepatocytes.....	59
Statistical analysis.....	59
Results and discussion.....	61
Chapter 3.....	62
Introduction.....	63
Attempts to increase the sensitivity of isolated hepatocytes.....	63
Percoll treatment of isolated hepatocytes..	66
Reduction of the duration of incubation....	66
Decreasing the concentration of hepatocytes in the final incubation volume.....	66
Effect of glucagon-related peptides on GCS flux.....	69
Is the stimulation of GCS by glicentin and oxyntomodulin brought about via interaction with the glucagon receptor?.....	75
Discussion.....	92
Chapter 4.....	97
Introduction.....	98
Protein phosphorylation and flux through GCS..	98
Effect of calcium-mobilizing hormones on GCS..	121
Role of protein kinase C in the regulation of	

GCS.....	132
Discussion.....	139
Unanswered questions.....	144
Chapter 5.....	146
Summary.....	147
References.....	149

LIST OF FIGURES

Figure	Page
1.1 Metabolism of glycine.....	3
1.2 Scheme for the overall reaction of GCS.....	11
1.3 Schematic representation of the preproglucagon molecule.....	21
1.4 Chemical structure of okadaic acid.....	33
1.5 Structures of the cAMP analogues, Sp-cAMPS and Rp-cAMPS.....	37
3.1 Glucagon stimulation of the flux through GCS and glycogenolysis.....	65
3.2 Stimulation of flux through GCS in isolated rat hepatocytes.....	72
3.3 Stimulation of glycogenolysis in isolated rat hepatocytes.....	74
3.4 Effect of GLP-1 on glucagon-stimulated GCS flux in isolated rat hepatocytes.....	78
3.5 Effect of GLP-1 on glucagon-stimulated glycogenolysis in isolated rat hepatocytes.....	80
3.6 Effect of miniglucagon on the flux through GCS and glycogenolysis in isolated rat hepatocytes.....	82
3.7 Inhibition of glucagon-stimulated GCS flux and glycogenolysis by des-His ¹ -Glu ⁹ glucagon amide..	85
3.8 Inhibition of oxyntomodulin-stimulated GCS flux and glycogenolysis by des-His ¹ -Glu ⁹ glucagon amide.....	87
3.9 Inhibition of glicentin-stimulated GCS flux and glycogenolysis by des-His ¹ -Glu ⁹ glucagon amide..	89
3.10 Inhibition of glucagon-stimulated GCS flux and glycogenolysis by des-His ¹ -Nle ⁹ -Ala ¹¹ -Ala ¹⁶ glucagon amide.....	91
4.1 Dose-response curve with okadaic acid.....	100
4.2 Effect of 10 nM okadaic acid on glucagon stimulation of flux through GCS in isolated hepatocytes.....	104
4.3 Effect of 10 nM okadaic acid on the glucagon stimulation of glycogenolysis in isolated hepatocytes.....	106
4.4 Effect of a maximal dose of okadaic acid on the glucagon-stimulated flux through GCS.....	108
4.5 Effect of a maximal dose of okadaic acid on the glucagon-stimulated glycogenolysis in isolated hepatocytes.....	110
4.6 Effect of Sp-cAMPS and Rp-8-Br-cAMPS on glycogenolysis and flux through GCS.....	113

4.7	Effect of Rp-8-Br-cAMPS on Sp-cAMPS stimulation of flux through GCS and glycogenolysis.....	116
4.8	Effect of Rp-8-Br-cAMPS on Sp-cAMPS on flux through GCS.....	118
4.9	Effect of Rp-8-Br-cAMPS on Sp-cAMPS stimulation of glycogenolysis.....	120
4.10	Effect of Rp-8-Br-cAMPS on the glucagon stimulation of glycogenolysis and GCS flux....	123
4.11	Effect of Rp-8-Br-cAMPS on glucagon stimulation of flux through GCS.....	125
4.12	Effect of Rp-8-Br-cAMPS on glucagon stimulation of glycogenolysis in isolated hepatocytes....	127
4.13	Effect of vasopressin on the flux through GCS And glycogenolysis in isolated hepatocytes....	129
4.14	Stimulation of the flux through GCS and glycogenolysis by angiotensin II in isolated rat hepatocytes.....	131
4.15	Effect of PMA on the flux through GCS and glycogenolysis in isolated hepatocytes.....	134
4.16	Effect of PMA on glucagon stimulation of flux through GCS.....	136
4.17	Effect of PMA on glucagon stimulation of glycogenolysis.....	138
4.18	Unanswered questions.....	145

LIST OF TABLES

Table	Page
3.1 Effect of Percoll purification of isolated hepatocytes on their sensitivity to glucagon stimulation of GCS flux and glycogenolysis.....	67
3.2 Effect of preincubation of hepatocytes on their sensitivity to glucagon stimulation of GCS flux and glycogenolysis.....	68
3.3 Effect of hepatocyte concentration on their sensitivity to glucagon stimulation of GCS and glycogenolysis.....	70
3.4 Effect of GLP-1 (7-36)amide on the flux through GCS and glycogenolysis in isolated hepatocytes.....	76
4.1 Effect of okadaic acid on the flux through GCS in isolated mitochondria.....	101
4.2 Effect of Sp-cAMPS and Rp-8-Br-cAMPS on the flux through GCS in isolated mitochondria.....	114

LIST OF ABBREVIATIONS

ADP.....	adenosine diphosphate
ATP.....	adenosine triphosphate
BSA.....	bovine serum albumin
cAMP.....	cyclic adenosine-3',5'-monophosphate
cAMPS.....	cyclic adenosine-3',5'-monophosphothiorate
DMSO.....	dimethyl sulfoxide
EGTA.....	ethylene glycol-bis (β -aminoethyl ether) N,N,N',N'-tetraacetic acid
EDTA.....	ethylenediaminetetraacetic acid
GCS.....	glycine cleavage enzyme system
GLP-1.....	glucagon-like peptide-1
GLP-2.....	glucagon-like peptide-2
Hepes.....	N-[2-hydroxyethyl]piperazine-N'-(2-ethanesulfonic acid]
MPF.....	major preproglucagon fragment
Miniglucagon...	glucagon (19-29)
NAD+.....	nicotinamide adenine dinucleotide (oxidized form)
NADH.....	nicotinamide adenine dinucleotide (reduced form)
NADP.....	nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH.....	nicotinamide adenine dinucleotide phosphate (reduced form)
PCA.....	perchloric acid
PMA.....	phorbol 12-myristate 13-acetate
PK-A.....	protein kinase A
PK-C.....	protein kinase C
PP1.....	protein phosphatase 1
PP2A.....	protein phosphatase 2A
PP2C.....	protein phosphatase
THF.....	tetrahydrofolate

CHAPTER 1

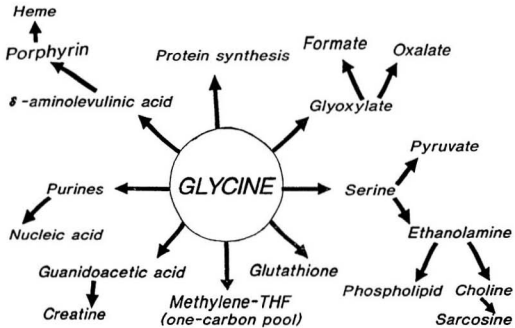
INTRODUCTION

INTRODUCTION

Glycine is a dietary nonessential amino acid that can be readily synthesized from common metabolic intermediates in all organisms. It is considered structurally the simplest of amino acids, but it has a complex pattern of metabolism. It is present in high concentrations in collagen and is abundant in most animal proteins. Apart from its role in protein synthesis, glycine plays multiple roles in many synthetic reactions as shown in Fig. 1.1.

Glycine is a glucogenic amino acid. It originally received its name, reminiscent of sugar, because it has a sweet taste. Conversion to serine is the dominant route for gluconeogenesis from glycine. Its conversion to aminoacetone and subsequently to pyruvate is another potential glucogenic route. However, this route does not appear to be important in mammals (Bender, 1985). Glycine is also a major source of one-carbon units with its α -carbon being passed to tetrahydrofolate to give methylene-tetrahydrofolate. Glycine condenses with succinyl-CoA to give δ -aminolevulinic acid, which is the precursor of heme, chlorophylls of plants and photosynthetic bacteria, and the cobalamins, notably vitamin B₁₂ and its derivatives. It is important for bile conjugation and in sarcosine, and glutathione synthesis. Glycine is very important in mammalian liver in the conjugation of foreign compounds taken in the diet. Benzoic acid derivatives are metabolized by conjugation

Fig. 1.1 Metabolism of glycine



with glycine to hippuric acid derivatives which are excreted in the urine. It serves in the detoxification of salicylates through the formation of salicyluric acid. In addition, it is very important in purine synthesis with its carbon incorporated at the number 4 and 5 positions of the purine ring and its nitrogen incorporated at the number 7 position. Glycine and arginine are used to form guanidoacetic acid which is converted to creatine. Furthermore, glycine is a major inhibitory neurotransmitter in the spinal cord and brain.

Under normal conditions, glycine is a nonessential amino acid. However, it has been proposed that glycine becomes indispensable in wound healing and repletion of tissue after depletion (Yu et al., 1985). Glycine is also present in most parenteral solutions in large quantities (Stegink et al., 1983).

ROUTES OF GLYCINE CATABOLISM

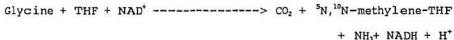
D-amino acid oxidase pathway

D-amino acid oxidase catalyses the conversion of glycine to glyoxylate and NH_3 . However, the high K_m of this enzyme for glycine suggests that this would make a minor contribution in the overall glycine catabolism (Neims and Hellarman, 1962). Glyoxylate may also be formed from glycine by transamination

(Nakada and Sund, 1958). In mammalian tissues there are two glycine aminotransferases: one linked to glutamate/ α -ketoglutarate and the other to alanine/ pyruvate as amino donor/ acceptor. Glycine is a very poor substrate for both of these enzymes and they appear to function in the direction of glycine synthesis rather than in the direction of glycine catabolism.

Glycine cleavage enzyme system

The bulk of glycine catabolism in vertebrates is known to occur by way of the hepatic glycine cleavage system (Yoshida and Kikuchi, 1972; 1973). In mammals, the glycine cleavage enzyme system (GCS) occurs in mitochondria. The overall reaction catalysed by the glycine cleavage system is:



Besides being a major pathway of glycine catabolism in mammalian tissues, this system is also an important source of ${}^5\text{N}, {}^{10}\text{N}$ -methylene-THF for a variety of synthetic reactions. Alternatively, the fate of methylene-THF may be to react with a second molecule of glycine to form serine and regenerate THF, a reaction catalysed by serine hydroxymethyltransferase. This scheme has been termed the "glycine cycle" (Snell, 1984).

Glycine cleavage system in different organisms

GCS was first described in cell-free extracts from the anaerobic bacterium *Diplocccus glycinophilus* (Sagers and Gunsalus, 1961). Richert et al., (1962) reported the occurrence of GCS in liver homogenates of pigeon, duck, and chicken which were shown to release the first carbon of glycine as CO₂.

A comparative study of glycine catabolism showed that GCS occurs in the livers of a variety of vertebrate species (Yoshida and Kikuchi, 1972). GCS was reported in the liver of human, pig, cow, dog, goat, rabbit, guinea pig, fish, amphibians, and reptiles. Birds and reptiles exhibit the highest activities among vertebrates, while rat possesses the highest among mammals.

Glycine cleavage system in rat

Kawasaki et al., (1966) were the first to report that rat liver mitochondria possess the glycine cleavage enzyme. Liver mitochondria could synthesize two molecules of glycine from one molecule each of serine, bicarbonate, and ammonia, which represented the reverse of the glycine cleavage reaction. Sato et al., (1969) were the first to report the reaction in the physiological direction of glycine cleavage. GCS activity was measured in different tissues of the rat by Yoshida and

Kikuchi, (1973). Among the tissues tested, the liver exhibited the greatest activity while lung, skeletal muscle, and small intestine had low or negligible activities.

GCS location and components

GCS is confined to the inner mitochondrial membrane of all the tissues that have been studied (Hiraga *et al.*, 1972; Hayasaka *et al.*, 1980). The enzyme is loosely associated with the inner mitochondrial membrane.

GCS components have been isolated from a number of bacteria (Klein and Sagers, 1966), plant (Walker and Oliver, 1986), and animal sources (Kikuchi and Hiraga, 1982). GCS consists of four different proteins which are as follows: 1) a pyridoxal phosphate-dependent glycine decarboxylase; 2) a lipoic acid-containing aminomethyl transferase; 3) ^{15}N , ^{18}N -methylene-tetrahydrofolate synthesising protein; and 4) a flavin-containing lipoamide dehydrogenase. These proteins are referred to as P-, H-, T- and L-proteins, respectively. The glycine cleavage enzyme complex requires the presence of all four proteins for its activity.

T-protein

It is a tetrahydrofolate-dependent enzyme which catalyses the degradation of H-protein-bound intermediate to ammonia and

methylene-THF. T-protein has been purified from the liver of rat and its relative molecular weight is 33,000 (Motokawa and Kikuchi, 1974).

L-protein

It is a lipoamide dehydrogenase, flavin-containing protein (Kikuchi, 1973). It functions to reoxidize lipoic acid back to the disulfide form through the transfer of reducing equivalents to NAD⁺. Lipoamide dehydrogenase is a homodimer with a subunit that has a relative molecular weight of about 55,000 (Carothers *et al.*, 1989).

P-protein

It is a homodimer with a relative molecular weight of about 210,000. It is a pyridoxal phosphate-dependent protein, responsible for the decarboxylation of the carboxyl carbon of glycine and the transfer of the aminomethyl remnant to the H-protein. P-protein is inactive by itself, it requires the presence of H-protein for its activity (Motokawa and Kikuchi, 1972).

H-protein

H-protein is a small, heat stable, acidic protein that contains lipoic acid as a prosthetic group (Motokawa and

Kikuchi, 1969 a,b; 1971). It acts as a carrier of the aminomethyl intermediate between the active sites of the P-protein and T-protein. The relative molecular masses of H-proteins, calculated from their sequences, are approximately 14,000 each when the lipoic acid group is included.

The functional glycine decarboxylase is an enzyme complex consisting of P- and H-proteins (Hiraga and Kikuchi, 1980a). P-protein activity was increased more than 100,000-fold by the addition of H-protein (Hiraga and Kikuchi, 1980b).

Mechanism of action

First, glycine and H-protein bind to the P-protein at separate sites before the release of any product (Fujiwara and Motokawa, 1983) with glycine forming a Schiff base with the carbonyl group of pyridoxal phosphate bound to P-protein (Hiraga and Kikuchi, 1980b). Decarboxylation results in the release of CO₂ (Fujiwara and Motokawa, 1983) and the generation of an H-protein intermediate. Then the methylene carbon of glycine is transferred to one of the sulphydryl groups of the lipoic acid prosthetic group of H-protein (Hiraga and Kikuchi, 1980a). The methylene carbon is attached to ⁵N,¹⁵N-methylene-THF in a reaction catalysed by T-protein (Fujiwara et al., 1984). The last reaction is the reoxidation

of the reduced lipoic acid of H-protein to its disulfide form. This step is catalysed by L-protein, which contains FAD as a cofactor, and involves the transfer of reducing equivalents to NAD⁺. The mechanism of GCS is illustrated in Fig. 1.2.

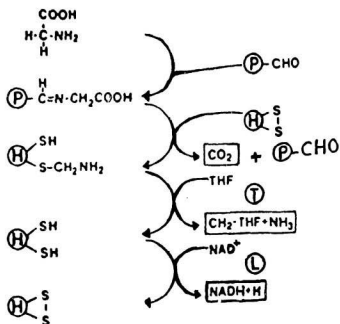
Regulation of GCS

Many different mechanisms have been proposed for the regulation of GCS.

Branched chain α -keto acid

Inhibition of GCS by branched chain α -keto acids was reported by O'Brien (1978) and Kochi *et al.*, (1986). They suggested that glycine decarboxylase and the branched chain α -keto acid dehydrogenase share a common subunit which is the lipoamide dehydrogenase and that these α -keto acids exert their effects by providing reducing equivalents to the glycine cleavage system, possibly through lipoamide dehydrogenase. However, the concentrations of the branched chain α -keto acids that were used in these experiments (2 and 5 mM) are much higher than the physiological concentrations which are 10-50 μ M (Schauder, 1984). Therefore, if this occurs it is only in pathological conditions such as the ketotic forms of hyperglycinemia as proposed by O'Brien (1978).

Fig.1.2 Scheme for the overall reaction of GCS



Oxidation-reduction in the mitochondrial pyridine nucleotides

It has been proposed that GCS, in isolated perfused rat liver or in intact rat liver mitochondria, is very sensitive to the oxidation-reduction state of the mitochondria (Hampson *et al.*, 1983; 1984). Metabolic flux through GCS in perfused rat liver was inhibited by processes that lead to reduction of the mitochondria's NAD(H) redox couple. Infusion of β -hydroxybutyrate or octanoate inhibited $^{14}\text{CO}_2$ production from l- ^{14}C glycine by 33 and 50%, respectively. On the other hand, infusion of acetoacetate, which increases NAD^+/NADH , stimulated GCS significantly and completely reversed the inhibition of $^{14}\text{CO}_2$ production by octanoate. In isolated rat liver mitochondria it was found that glycine oxidation was stimulated in state III when compared to state IV and was maximal in the uncoupled state (Hampson *et al.*, 1983). Alternatively, respiratory inhibitors such as rotenone and reducing substrates such as succinate, α -ketoglutarate, etc., greatly inhibited the glycine decarboxylation. In addition, direct measurement of mitochondrial pyridine nucleotides showed that the flux through GCS was correlated with changes in both the NAD(H) and NADP(H) redox couples.

However, Schauder (1984) reported that feeding a high protein diet leads to an increase in the flux through GCS and is accompanied by an increase in the levels of branched chain

α -oxo acid and a decrease in the mitochondrial NAD^+/NADH ratio, both of which would be expected to decrease GCS activity according to Hampson *et al.*, (1983). In addition, Jois *et al.*, (1989) suggested that glucagon stimulates the flux through GCS. This stimulation cannot be explained by oxidation-reduction states of mitochondria because glucagon results in "reduced redox states" of both the cytosolic and mitochondrial pyridine nucleotides (Sugano *et al.*, 1980; Balaban and Blum, 1982).

Dietary protein

Ishikawa, (1976) and Fafournoux, (1990), reported a large increase in hepatic uptake of glycine after feeding a high protein diet. However, despite this increase in the hepatic uptake of glycine, its concentration decreased in the liver (Fafournoux, 1990). This suggested a primary activation of intrahepatic glycine metabolism.

Glucagon stimulates the flux through GCS (Jois *et al.*, 1989). Ewart *et al.*, (1992) reported that the stimulation of GCS by high protein feeding has similar characteristics to that elicited by glucagon. Thus, they proposed that the high circulating glucagon concentration found after ingestion of a high protein meal (Robinson *et al.*, 1981) may be an important signal in stimulating GCS. It was pointed out by Ewart *et al.*,

(1992) that ingestion of a high protein diet leads to ingestion of a large amount of glycine which will lead to increased availability of free amino acids. Therefore, the excess glycine must be catabolized, stimulating the flux through GCS. Rats fed 15%-casein meal (normal-protein diet) oxidized glycine at a rate less than 0.15 nmoles/ min/ mg, whereas for rats fed on 60%-casein diet (high-protein meal) the rate was 0.38 nmoles/ min/ mg or higher (Ewart *et al.*, 1992). It has also been reported that glycine catabolism is stimulated in normal protein-fed rats when they ingest a single high-protein meal 2 hours before being sacrificed, illustrating the rapid response of GCS to high protein intake.

Hormonal regulation

Many studies have shown that the flux through GCS is regulated by several hormones, by hormones known to act via cAMP as well as by hormones known to act by increasing the intracellular calcium concentration. Jois *et al.*, (1989) were the first to report stimulation of the flux through GCS by glucagon. Incubation of isolated hepatocytes with glucagon resulted in the stimulation of the flux through GCS in a dose-dependent manner, with a maximum stimulation occurring at 100 nM glucagon. The stimulation of the flux through GCS by glucagon was also evident in mitochondria isolated from rats

given glucagon, intraperitoneally, 25 minutes before being sacrificed. Dibutyryl-cAMP was also equally effective in stimulating the GCS flux and a significant correlation was observed between increased cellular cAMP levels induced by glucagon and stimulation of the flux through GCS by glucagon (Jois *et al.*, 1990a). Jois *et al.*, (1989) suggested two mechanisms of action of glucagon on glycine catabolism in liver. One is covalent modification, by phosphorylation, which occurs by activation of the cAMP-stimulated protein kinase. Other known target enzymes for this mechanism include glycogen phosphorylase, phenylalanine hydroxylase, and pyruvate kinase (Garrison *et al.*, 1984). These effects are labile in that they are readily reversible (via phosphatases) upon removal of the hormone. The second type of glucagon action is the stimulation of mitochondrial metabolism of a variety of substrates. This effect appears to be fairly stable in that it persists for some time after the removal of the hormone and remains evident in mitochondria that have been isolated and washed without any precautions taken to preserve the phosphorylation state of proteins (Halestrap, 1986). However, a link between changes in intramitochondrial functions and increased cytoplasmic cAMP has not been established. Jois *et al.*, (1989) suggested that glucagon stimulation of the flux through GCS belongs to the second type of action since the effect is long and persists in

mitochondria isolated from glucagon injected rats.

It has been suggested that hepatocytes possess two distinct receptors for glucagon, a GR-1 receptor coupled to stimulation of inositol phospholipid breakdown and a GR-2 receptor coupled to stimulation of adenylate cyclase activity (Wakelam *et al.*, 1986). This suggestion will be examined later in this thesis. However, Jelinek *et al.*, 1993 isolated a complementary DNA clone for the glucagon receptor by an expression cloning strategy, and the receptor protein was expressed in several kidney cell lines. The cloned receptor bound glucagon and caused an increase in the intracellular concentration of cAMP and transduced a signal that led to increase in the intracellular calcium concentration. No second glucagon receptor was detected by Jelinek *et al.*, (1993). Therefore, they suggested that there is only one glucagon receptor. It may be similar to the calcitonin and parathyroid hormone receptors which can transduce signals leading to the accumulation of two different messengers, cAMP and calcium.

The flux through GCS, in isolated perfused rat liver, is also stimulated to 100-200% above the basal rate by 1 μ M epinephrine, 1 μ M norepinephrine, or 100 nM vasopressin (Jois *et al.*, 1990b; Brosnan *et al.*, 1990). These hormones are known to exert their effects in the liver by increasing the free intracellular calcium concentration. Jois *et al.*, (1990b)

related the fact that glycine is a gluconeogenic amino acid and that glucagon, catecholamine and vasopressin have been shown to increase hepatic glucose output by stimulating glycogenolysis and gluconeogenesis. Thus, it is physiologically important that these hormones stimulate the flux through GCS. It has also been reported that the flux through GCS is sensitive to concentrations of calcium which would be achieved in the cytoplasm of hepatocytes stimulated by calcium-mobilizing hormones (Jois *et al.*, 1990b). Although, exclusion of calcium from the incubation medium reduced the basal flux through GCS in isolated hepatocytes, it did not affect the degree of stimulation of flux through the GCS by glucagon. Therefore, the ability of glucagon to stimulate flux through the GCS was independent of the presence of calcium in the medium (Jois *et al.*, 1990b).

DEFECTS IN GLYCINE CATABOLISM

Hyperglycinemia occurs in a series of syndromes characterized by diminished capacity to catabolize glycine due to reduced activity of the glycine cleavage system. Nyhan *et al.*, (1961) were the first to report the clinical symptoms of hyperglycinemia. Its symptoms were lethargy, convulsive seizures, prolonged episodes of vomiting, ketoacidosis and abnormally high levels of glycine in the plasma and urine.

Hyperglycinemia is divided into two distinct diseases, ketotic and nonketotic hyperglycinemia.

Ketotic hyperglycinemia appears to be a secondary consequence of genetic disorders in other metabolic pathways, particularly those for the branched chain amino acid and α -keto acids. It has also been observed to accompany D-glycemic acidemia (Kolvaara *et al.*, 1979), experimental models of phenylketonuria (Isaacs and Greengard, 1980) and the use of the antiepileptic drug valproic acid (Jaeken *et al.*, 1977). Several mechanisms have been proposed to explain this syndrome. Direct inhibition of the GCS by different metabolites was suggested by O'Brien, (1978).

Nonketotic hyperglycinemia, which represents the other type of hyperglycinemia, is an inborn error of amino acid metabolism in which large amounts of glycine accumulate in body fluids. The concentration of glycine is particularly high in the cerebrospinal fluid; it was reported to be ten times higher than in normal subjects. Most patients are severely mentally retarded and have seizure disorders. Convulsive seizures and neuropenia have been found in most cases of nonketotic hyperglycinemia. This syndrome is caused by a rare autosomal recessive gene. Patients with a defect in P-, T-, or H-protein have been reported (Hiraga, *et al.*, 1981).

GLUCAGON-RELATED PEPTIDES

The mammalian glucagon precursor (proglucagon) is a 180 amino acid peptide. It is principally expressed both in the cells of the islets of Langerhans and in the L-cells of the intestinal mucosa (Novak *et al.*, 1987; Mojsov *et al.*, 1986; Orskov *et al.*, 1986; 1989). The proglucagon gene is also expressed in selected neurons of the brain (Holst *et al.*, 1987).

Molecular cloning of the hamster preproglucagon cDNA (Bell *et al.*, 1983a) and of the human glucagon gene (Bell *et al.*, 1983b) revealed that it coded for peptides other than glucagon. The first 20 amino acids form the leading sequence of the preproglucagon molecule. In the pancreatic cells, the major preproglucagon-products are glucagon and major preproglucagon fragment (MPF) which contains both glucagon-like peptide-1 (GLP-1) and glucagon-like peptide-2 (GLP-2) sequences (Mojsov *et al.*, 1986; Orskov *et al.*, 1992). In the intestine, the preproglucagon-derived peptides include glicentin, oxyntomodulin and GLP-1 and GLP-2. GLP-1 is further truncated in the intestine to form GLP-1 (7-37) and GLP-1 (7-36)NH₂ (Mojsov *et al.*, 1986; 1990; Holst *et al.*, 1987; Suzuki *et al.*, 1992), which are equipotent in insulinotropic activity (Fehmann *et al.*, 1989; Siegel *et al.*, 1992 and Gefel *et al.*, 1990). The cleavage of the preproglucagon molecule is

illustrated in Fig. 1.3.

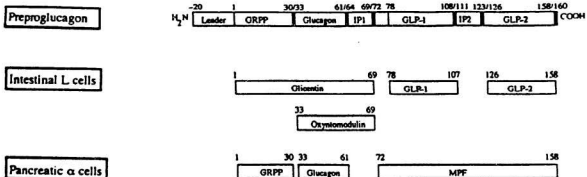
Oxyntomodulin

Oxyntomodulin is a 37-amino acid peptide isolated from porcine jejunum-ileum (Bataille *et al.*, 1982a). It is a circulating hormone that is released from the gut during digestion. The level of oxyntomodulin increased by a factor of two when rats were refed for 2 hours after a 12-hours fasting, reaching 23.3 ± 3.0 pmol/L (Kervran *et al.*, 1987). It displays tissue specificity contrasting with that of glucagon, which possesses biological activities directed towards the tissues implicated in fuel homeostasis. Oxyntomodulin's main target tissue is the gastric mucosa (Bataille *et al.*, 1981), where it inhibits gastric acid secretion at doses that are 15-fold lower than the effective doses of glucagon in the anesthetized (Duberasquet *et al.*, 1982), or conscious rat (Jarrousse *et al.*, 1985; 1986), as well as in humans (Schjoldager *et al.*, 1988; 1989). Oxyntomodulin contains the glucagon sequence extended by a C-terminal basic octapeptide: Lys-Arg-Asn-Lys-Asn-Asn-Ile-Ala (Bataille *et al.*, 1982b). The molecular basis for oxyntomodulin specificity resides in the COOH-terminal octapeptide, which differentiates it from glucagon. This octapeptide mimics the biological activity of the hormone. It was shown to inhibit histamine-, milk meal-,

Fig. 1.3

Schematic representation of the preproglucagon molecule

(Thornes and Waeber, 1993)



or pentagastrin-stimulated gastric secretion in conscious rats provided with chronic fistulae (Jarrousse *et al.*, 1985; 1993; Carles-Bonnet *et al.*, 1992). The mode of action of oxyntomodulin on gastric mucosa is controversial. Although a good inhibitor of gastric acid secretion it increases the adenosine 3',5'-cyclic monophosphate levels in isolated fundic glands (Bataille *et al.*, 1981; 1988), a characteristic shared by histamine, which is a major stimulant of gastric acid secretion (Code, 1982).

It has also been reported that oxyntomodulin stimulated insulin release monophasically in the presence of low (6 mM) medium glucose concentration. Furthermore, oxyntomodulin potentiated glucose-induced insulin release (10 mM glucose) in a dose-dependent manner, although it was less powerful than similar concentrations of glucagon (Jarrousse *et al.*, 1984). The effects of glucagon and oxyntomodulin on blood glucose level were compared in rat. During the course of glucagon infusion, at a dose of 1.8 nmol/ Kg, blood glucose was increased 2-fold while the same dose of oxyntomodulin induced only a small, although significant, increase over the control basal values. It was necessary to increase the oxyntomodulin dose 10-fold to produce the same degree of hyperglycemia as that induced by glucagon (Kervran *et al.*, 1990).

GLP-1

GLP-1 is an intestinally derived hormone from the glucagon family of peptides. GLP-1 is found in four variants: proglucagon- 78-108, GLP-1 (7-37), proglucagon-72-108, or GLP-1 (1-37), and their respective amidated forms. Purification of GLP-1 from human and pig intestine and its analysis revealed that the major naturally occurring peptide corresponds to proglucagon (78-107) NH₂, or GLP-1 (7-36) amide (Kreymann *et al.*, 1988; Orskov *et al.*, 1989).

GLP-1 secretion is stimulated by ingestion of a mixed meal and its plasma concentration varies with the meal pattern (Elliot *et al.*, 1993; Orskov *et al.*, 1994). GLP-1 levels increase from 1-10 pmol/L to 20-50 pmol/L after ingestion of a mixed meal (Orskov *et al.*, 1994). Significant increases in GLP-1 levels may occur after a few minutes, and peak values may be reached 15-30 minutes after oral intake of a stimulus.

GLP-1 (7-36) amide has profound effects on the endocrine pancreas in mammals. In physiological concentrations, GLP-1 stimulates insulin (Mojsov *et al.*, 1987; Holst *et al.*, 1987; Orskov and Poulsen, 1991) and somatostatin secretion and strongly inhibits glucagon secretion (Holst *et al.*, 1987; Orskov *et al.*, 1988). Its effect on insulin secretion is glucose-dependent. However, GLP-1 infused intravenously in physiological amounts enhances insulin secretion significantly

at euglycemia (in the fasting and/ or postabsorptive state) in humans (Orskov *et al.*, 1993; Hvidberg *et al.*, 1994). Not only does GLP-1 stimulate insulin release (Weir *et al.*, 1989), but it also stimulates the expression of the proinsulin gene and proinsulin synthesis (Fehmann and Habener, 1991). The half-life of GLP-1 infused intravenously into humans is about 5 minutes, and the metabolic clearance rate has been calculated to be approximately 13 ml/ Kg/ min (Orskov *et al.*, 1993). Therefore, the peptide seems to be rapidly and effectively removed from the circulation. The kidneys seem to play a role in this process since GLP-1 was efficiently extracted by isolated perfused rat kidneys (Ruiz-Grande *et al.*, 1993). Orskov *et al.*, (1992) have reported high levels of plasma GLP-1 in patients with renal failure indicate the kidneys contribute to GLP-1 elimination, *in vivo*.

Expression cloning of the pancreatic β -receptor of GLP-1 has been reported recently (Thornes, 1992; Thornes and Waeber, 1993). The receptor belongs to the seven-transmembrane, G-protein coupled superfamily of receptors. Its affinity for GLP-1, as identified by binding to cloned receptors transfected into various cell lines, corresponds to an affinity constant approximately 10^{-9} mol/ L, and the binding is highly specific for GLP-1 (Hjorth *et al.*, 1994). Glucagon may bind to the receptor but with affinity at least 100-fold

less than GLP-1, while none of the peptides of the glucagon-secretin family bind to the receptor (Holst, 1994). Extrapancreatic GLP-1 receptors have been identified in rat brain, kidney, and skeletal muscle (Wheeler *et al.*, 1993). Kanse *et al.*, (1988) reported displaceable binding of GLP-1 (7-36) amide to homogenates of rat lung and brain.

The interest in GLP-1 with respect to diabetes mellitus is because of its effects on insulin secretion and its ability to lower blood glucose. In patients with type II diabetes, infusion of GLP-1 eliminated postprandial glucose excursions for 60 minutes after a mixed meal (Nathan *et al.*, 1992). In patients whose blood glucose levels were regulated, near physiological amounts of GLP-1 infused during ingestion of a mixed meal nearly eliminated insulin requirements in these diabetic patients (Gutniak *et al.*, 1992). Moreover, in poorly controlled patients with type II diabetes, a GLP-1 infusion of 1.2 pmol/ kg/ min completely normalized blood glucose levels within 2-4 hours (Nauck *et al.*, 1993)

Glicentin

Glicentin has been isolated from porcine intestinal mucosa (Sundby *et al.*, 1975; Larsson and Moody, 1980). It has been suggested that glicentin is a prohormone to glucagon (Jacobsen *et al.*, 1977) or to other fragments with glycogenolytic

properties (Holst, 1978). The circulating level of plasma glicentin reaches approximately 1 nM following nutrient ingestion (Ohneda, 1987), a concentration which is far higher than concentrations of the other gastrointestinal hormones.

It has been reported that the glucagon-secreting A cells of the pancreas contain a glicentin-like peptide which is located in the secretory granules (Moody *et al.*, 1977; Ravazzola *et al.*, 1979 a,b). The physiological action of glicentin is not completely understood. Glicentin was found to have no effect on basal insulin release in the mouse, it inhibited glucose-induced insulin secretion (Ahren and Lundquist, 1980). Recently, Ohneda *et al.*, (1995) have demonstrated an insulintropic action of glicentin on pancreatic β -cells in dogs. Although these effects of glicentin have been reported, the actual mechanism of its action remains to be investigated.

Miniglucagon

It has been shown that interaction of glucagon with liver cells leads to the cleavage of the hormone by an endopeptidase giving rise to the local production of two COOH-terminal fragments (Mallat *et al.*, 1987; Blache *et al.*, 1989), glucagon (19-29), referred to as miniglucagon by Unger and Orci, (1990), and glucagon (18-29). Glucagon (19-29) is present in

rat pancreas and stomach; its tissue concentration corresponds to about 3% of that of glucagon but there are no detectable amounts of the peptide in rat plasma (Blache *et al.*, 1990). It has been shown that glucagon (19-29) is generated upon incubation of glucagon with liver plasma membranes and was degraded with a half-life of < 10 seconds (Blache *et al.*, 1990) which may explain why, unlike glucagon, glucagon (19-29) is not found in the circulation. Rather if it is physiologically active it must be produced locally at its site of action. The enzyme, or enzymatic system, responsible for miniglucagon production is a thiol endopeptidase. Blache *et al.*, (1990) reported that miniglucagon production was inhibited by thiol-reactive agents such as para-chloromercuribenzoate, N-ethylmaleimide and para-chloromercuribenzenesulfonate. Partial inhibition by the chelating agent 1, 10-phenanthroline suggests that the enzyme also includes a catalytically active metal, which contributes to the full expression of the enzymatic activity.

Glucagon (19-29) and glucagon (18-29), at nanomolar concentrations, inhibited the activity of the plasma membrane calcium pump of liver plasma membranes which is responsible for the active extrusion of calcium from the cell (Blache *et al.*, 1990). Miniglucagon exerted a biphasic stimulation on this system in liver (Lotersztajn *et al.*, 1990) where it was

1000 times more effective than glucagon itself. Both peptides have no effect on adenylyl cyclase activity, but it has been reported that their action on the calcium pump is mediated by cholera toxin-sensitive G protein(s) (Lotersztajn *et al.*, 1990). The demonstration that glucagon itself can be processed into a fragment which displays an individual biological specificity, namely the regulation of the membrane-bound calcium pump, raises the question of the presence and nature of receptors for this peptide on liver plasma membrane. Any metabolic actions of miniglucagon *in vivo* remain to be elucidated.

GLUCAGON RECEPTOR ANTAGONISTS

Increasing interest in the role of glucagon in diabetes in the maintenance of elevated blood glucose levels has made the development of glucagon antagonists very important. Furthermore, a pure antagonist of glucagon would be a valuable tool for investigating the mechanism of its biological actions. Earlier structure-function studies have been directed at elucidating the functional groups and the conformational features of the hormone that are responsible for recognition and binding from those that transduce the biological response.

Des-His¹-[Glu⁹]-glucagon amide and its biological activities

Des-His¹-[Glu⁹] glucagon amide was proven to be a relatively potent competitive antagonist to glucagon in hepatocyte membranes (Unson *et al.*, 1987). Its binding to the receptor, as measured by competitive displacement of ¹²⁵I-labeled glucagon from liver membranes, was approximately 40% as effective as glucagon itself. Moreover, this analogue did not activate adenylate cyclase or generate cAMP at any of the concentrations tested.

Post *et al.*, (1993) investigated the activities of des-His¹-[Glu⁹] glucagon amide compared to glucagon in modulating the activity of adenylyl cyclase in isolated intact and saponin-permeabilized canine hepatocytes. The antagonist, at 30 nM, was able to inhibit by 50% the cAMP accumulation induced by 3 nM glucagon. In saponin-permeabilized hepatocytes, the antagonist at about 100 nM inhibited by 50% the stimulation of adenylyl cyclase that was induced by 10 nM glucagon. In both intact and saponin-permeabilized hepatocytes the antagonist was without effect on the basal activity of adenylyl cyclase. These results identify the analogue des-His¹-[Glu⁹] glucagon amide as a true antagonist of glucagon action in both experimental preparations *in vitro*.

In vivo effects of the antagonist were reported by Unson *et al.*, (1989). Des-His¹-[Glu⁹] glucagon amide did not

appreciably stimulate glycogenolysis in fasted rabbits at concentrations more than 400 times the concentration of glucagon required for a measurable effect. Moreover, when the analogue was administered intravenously to normal rabbits in a mixture with the natural hormone at a ratio of 100:1 it was able to suppress almost completely the hyperglycemic effect of the added glucagon. In streptozotocin-induced diabetic rats the antagonist caused a 60-70% decrease in blood glucose (Unson *et al.*, 1989).

Des-His¹-[Nle⁹-Ala¹¹-Ala¹⁶] glucagon amide

In an effort to understand and maximize the structural features that contribute to glucagon receptor antagonism, many analogues of glucagon were synthesized incorporating the des-His¹-[Glu⁹] and the COOH-terminal amide substitutions. Their ability to compete with glucagon for receptor binding and adenylate cyclase activation were measured. Unson and Merrifield, (1994) speculated that a serine residue might cooperate with His¹ and Asp⁹ to produce an active center. Such a putative His, Asp, Ser active site would be reminiscent of the serine protease triad. Glucagon contains four serine residues at positions 2, 8, 11, 16, which are conserved among the members of the family. They tested for the requirement for serine by a series of substitutions for the hydrophilic

hydroxyl group in each of the four positions. Whereas neither serine 2, 11, nor 16 are required for receptor recognition, they showed that serine 16 is essential for signal transduction and suggested that it may be the third residue in glucagon to participate in the putative catalytic triad (together with aspartic acid and histidine 1) in the transduction of glucagon's response. Unson and Merrifield, (1994) applied computer graphics to this problem. Using the working model of glucagon in dilute solutions, they found that His¹, Asp⁹, and Ser¹⁶ side chains could be juxtaposed to create the hypothetical charge relay triad. They suggested that the glucagon-receptor complex acquires enzyme activity upon its formation and that this activity might initiate the transduction process.

When glutamic acid or the hydrophobic amino acids leucine or norleucine were substituted for aspartic acid at position 9 and 21 analogues that bound well with partial or no adenylate cyclase activation were produced (Unson *et al.*, 1991). Alanine at position 11 was reported to increase receptor binding affinity nearly 5-fold (Unson *et al.*, 1994). Consequently, derivatives incorporating these amino acid substitutions were synthesized and combined with the deletion of histidine at position 1 (Unson *et al.*, 1989; 1994). Among these analogues was des-His¹-[Nle⁹-Ala¹¹-Ala¹⁶] glucagon amide

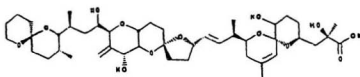
which bound as well or better than glucagon itself.

OKADAIC ACID

Okadaic acid is a polyether derivative of a 38-carbon fatty acid originally isolated from the black sponges *Halichondria okadai* and *Halichondria melanodocia* (Tachibana *et al.*, 1981). Its structure is given in Fig. 1.4. As a marine toxin, it is implicated as the causative agent of diarrhoeic shellfish poisoning, (DSP). It is known to be one of a family of related toxins including dinophysistoxin-1 and acanthifolicin. Such toxins are synthesized by dinoflagellates (marine plankton), especially of the genus *Dinophysis*, but they accumulate in organisms further up the food chain, including sponges, shellfish and, ultimately, humans, causing DSP (Hardie *et al.*, 1991). The first insight into its mechanism of action came from observations that it enhanced the contraction of isolated vascular smooth muscles from human umbilical arteries and rabbit aorta (Cohen and Cohen, 1989). Later, it was shown that it is a potent and specific inhibitor of protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) (Takai *et al.*, 1987; Ishihara *et al.*, 1989).

Okadaic acid has been shown to act as a tumour promoter in the mouse skin bioassay, but unlike many other tumour promoters it does not activate protein kinase C (Suganuma *et*

Fig. 1.4 Chemical structure of okadaic acid



Okadaic Acid

al., 1988). Because PP1 and PP2A are likely to be the chief enzymes that reverse the action of protein kinase C, it is not surprising that okadaic acid should be as potent a tumour promoter as the phorbol esters which activate protein kinase C. Tumour promotion probably stems from increased phosphorylation of one or more proteins that are regulated by protein kinase C and are dephosphorylated by PP1/PP2A.

Okadaic acid is a valuable tool to test the role of protein phosphorylation in any physiological response in intact cells since it is cell-permeable. PP1 and PP2A are two of the four major protein phosphatases in the cytosol of mammalian cells that dephosphorylate serine and threonine residues (Cohen and Cohen, 1989). Protein phosphatases are subdivided into two types with three subtypes of type 2. PP1 dephosphorylates the β -subunit of phosphorylase kinase specifically. PP2 dephosphorylates the α -subunit of phosphorylase kinase preferentially. The mechanism of phosphatase inhibition by okadaic acid is unknown. Remarkably, the sensitivity of PP1 and PP2A to okadaic acid has been conserved, and is identical in organisms as different as mammals, yeast, fruit flies, starfish, and higher plants (Cohen and Cohen, 1989; Cohen et al., 1990).

Okadaic acid as a probe for identifying biological processes that are controlled by phosphorylation

Since okadaic acid is hydrophobic, it can enter isolated intact cells and help reveal physiological processes that are controlled by phosphorylation/ dephosphorylation mechanisms. Okadaic acid produces a marked increase in the phosphorylation of many proteins in adipocytes and hepatocytes (Haystead *et al.*, 1989). Many of these proteins are cytosolic enzymes of glucose and lipid metabolism, such as acetyl CoA carboxylase, ATP-citrate lyase, 6-phosphofructo-2-kinase/ fructose-2,6-bisphosphatase and pyruvate kinase. Phosphorylation of glycogen synthase and glycogen phosphorylase is increased in the hepatocyte glycogen fraction.

Specificity of okadaic acid

All effects of okadaic acid on intact cells are maximal at 1 μ M, which is similar to the concentration of PP1 and PP2A *in vivo* (Haystead *et al.*, 1989). Okadaic acid specificity is emphasized by its failure to inhibit protein phosphatase 2C (PP2C), mitochondrial pyruvate dehydrogenase phosphatase, protein tyrosine phosphatases, acid and alkaline phosphatases and inositol triphosphatase (Bialojan and Takai, 1988). It has no effect on other phosphatases such as the other two major protein-serine/ threonine phosphatases, the Ca^{2+} /calmodulin-

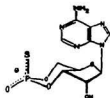
dependent protein phosphatase 2B (PP2B) and the Mg^{2+} -dependent PP2C (Bialojan and Takai, 1988). Okadaic acid has no effect on a variety of protein kinases such as cAMP or Ca^{2+} /calmodulin-dependent protein kinases, or protein kinase C (Takai *et al.*, 1987; Haystead *et al.*, 1989).

CYCLIC AMP ANALOGUES

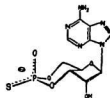
In eukaryotic cells, cAMP is a major intracellular second messenger that mediates the effects of hormonal or nerve stimulation. Since cAMP has poor cell permeability and is readily hydrolyzed by phosphodiesterase a number of analogues with better cell permeability and a longer biological life were used in intact cells (Parker-Botelho *et al.*, 1988). Cyclic adenosine-3',5'-monophosphothiorate, cAMPS, is an analogue of cAMP in which one of the two exocyclic oxygen atoms in the cyclic phosphate moiety is replaced by sulphur. Equatorial or axial thio substitution leads to R- and S-isomers, respectively (Fig. 1.5).

The Sp-diastereomer, Sp-cAMPS, is a cAMP-dependent protein kinase agonist which binds to the holoenzyme with approximately 10% of the binding affinity of cAMP and activates the enzyme half maximally at $0.3 \mu M$ (Rothermel *et al.*, 1983). Rp-cAMPS is a cAMP-dependent protein kinase antagonist and the first reported cAMP analogue which competes

**Fig. 1.5 Structures of the cAMP analogues
Sp-cAMPS and Rp-cAMPS**



Sp-cAMPS



Rp-cAMPS

with cAMP for binding sites on the regulatory subunit without causing dissociation of the holoenzyme (deWit *et al.*, 1982). This is attributed to the inability of this compound to induce the regulatory subunit conformational change necessary to release the catalytic subunit. Since the Sp-isomer behaves as a cAMP agonist, it was suggested that the holoenzyme dissociation depends on the formation of a salt bridge between a positively charged amino acid side chain and the equatorial exocyclic negatively charged oxygen which has been replaced by a sulphur atom in Rp-cAMPS (deWit *et al.*, 1982).

The Sp- and Rp-diastereomers of adenosine cyclic-3',5'-monophosphorothioate, Sp-cAMPS and Rp-cAMPS, are useful tools for studying cAMP-dependent control of many rate-limiting enzymes in the liver. Both analogues enter isolated hepatocytes readily and are resistant to the action of intracellular phosphodiesterases within the time frame of most experiments (Rothermel *et al.*, 1983; Meserve *et al.*, 1986). Therefore, intracellular concentrations of the analogues are constant throughout an experiment and production of metabolites is obviated. In hepatocytes isolated from fed rats, Rp-cAMPS inhibits the Sp-cAMPS-induced (Rothermel *et al.*, 1983) and glucagon-induced activation of cAMP-dependent protein kinase and the consequent effects of phosphorylation of the enzymes controlling both the glycogenolytic cascade

(Rothermel *et al.*, 1984a). Meserve *et al.*, (1986) reported inhibition of hepatic gluconeogenesis by Rp-cAMPS.

Specificity of Rp-cAMPS

Several observations suggest that Rp-cAMPS is specific for cellular cAMP-dependent protein kinase and that it has no other intrinsic biochemical properties. First, Rp-cAMPS does not activate phosphodiesterase nor is it metabolized by phosphodiesterases, ruling out the production of active metabolites (Jarvest *et al.*, 1982; Van Haastert *et al.*, 1983). Second, Rp-cAMPS does not affect the basal levels of cAMP-dependent protein kinase (Rothermel *et al.*, 1983; 1984b), phosphorylase, pyruvate kinase, and glycogen synthase (Rothermel *et al.*, 1984b). Finally, cAMP-dependent protein kinase is the only physiologically significant cAMP-binding protein in mammalian cells (Schwoch and Helz, 1977), other than phosphodiesterase, making it the most likely target for Rp-cAMPS.

CALCIUM-MOBILIZING HORMONES

Calcium-mobilizing hormones such as vasopressin and angiotensin II induce responses in hepatocytes and other cells through activation of a guanine nucleotide-binding protein (G protein) that regulates phosphatidyl inositol 4,5 bisphosphate

(PIP₂) breakdown (Cereba et al., 1983; Thomas et al., 1983; 1984; Berridge, 1984; Exton, 1988). The result is the generation of inositol 1,4,5-triphosphate (IP₃) and 1,2-diacylglycerol, which is formed concomitantly with IP₃ and has previously been shown to activate a Ca²⁺- and phospholipid-dependent protein kinase C (Kishimoto et al., 1980; Nishizuka, 1984a,b). These hormones act on the intracellular calcium concentration and glycogen phosphorylase partly by releasing Ca²⁺ from internal stores (Exton, 1981; Reinhart et al., 1984) and by augmenting the entry of extracellular Ca²⁺ (Keppens et al., 1977; Reinhart et al., 1984; Mauger et al., 1984).

Vasopressin is known to stimulate glucose output in rat liver (Hems and Whitton, 1973; Hems et al., 1976). It stimulates glycogen breakdown and gluconeogenesis in the rat liver at concentrations (0.1-1.0 ng/ml) which occur *in vivo*. This hepatic action of vasopressin, is important *in vivo*, in conditions where plasma vasopressin levels are high such as haemorrhagic shock (Kirk and Hems, 1974). Vasopressin causes activation of liver glycogen phosphorylase, the rate limiting enzyme for glycogenolysis (Keppens and DeWulf, 1976). Stubbs, et al., (1976) have shown that the action of vasopressin is very sensitive to extracellular calcium and is also partially diminished in the absence of extracellular K⁺ and she was the

first to suggest that cations and calcium in particular are likely to be implicated in vasopressin action on liver glycogen metabolism.

Experiments *in vivo* and *in vitro* showed that angiotensin II shares the glycogenolytic properties of vasopressin, both for the intact rat and for the perfused liver (Hems and Whitton, 1973; Ma and Hems, 1975; Keppens and DeWulf, 1975; Hems *et al.*, 1975). Keppens and DeWulf, (1976) reported that the concentrations of angiotensin II found to affect liver glycogen phosphorylase are likely to occur during haemorrhagic shock. A half-maximal activation of glycogen phosphorylase was obtained in isolated hepatocytes at about 0.3 ng angiotensin II/ ml. It has also been reported that moderate haemorrhage shock (withdrawal of 14-26 ml blood/ Kg) results in an increase of the concentration of angiotensin II to 0.33 ng/ ml blood in dogs. Stimulation of gluconeogenesis by vasopressin, and angiotensin II has been reported in hepatocyte suspensions (Whitton *et al.*, 1978). The hepatic glycine cleavage system has been reported to be stimulated by vasopressin (Brosnan *et al.*, 1990; Jois *et al.*, 1990b).

Stimulation of glucose output by vasopressin and angiotensin II in the liver cells is critically dependent on the calcium concentration (Stubbs *et al.*, 1976; Whitton *et al.*, 1978). Vasopressin was found to have no effect on

adenylyl cyclase activity. No increase in cAMP was detected in the liver or perfusate in response to vasopressin concentrations that were maximal or supramaximal with regard to their effect on glucose output (Kirk and Hems, 1974). Hems et al., (1978) found no increase in the hepatic content of cAMP or cGMP was after the addition of vasopressin or angiotensin II. Neither vasopressin nor angiotensin II affects protein kinase A activity in the liver (Keppens and DeWulf, 1975; 1976).

However, the calcium messenger and cAMP systems are to some extent interdependent in mammalian liver. Vasopressin stimulation of inositol phosphate accumulation in isolated hepatocytes is enhanced by cyclic AMP-dependent kinase but inhibited by protein kinase C (Pittner and Fain, 1990). In contrast, down regulation of protein kinase C significantly enhanced the maximal phosphoinositide response due to vasopressin. Mauger et al., (1985) showed that in rat hepatocytes, cAMP- and Ca^{2+} -linked hormones increase synergistically the Ca^{2+} influx by activating the same Ca^{2+} gating system. It has also been suggested that glucagon facilitates the action of vasopressin on intracellular Ca^{2+} concentration by accelerating the release of Ca^{2+} from the internal store and potentiating the vasopressin-mediated Ca^{2+} influx (Combettes et al., 1986). Vasopressin and angiotensin

II also inhibit the accumulation of cAMP in isolated hepatocytes stimulated by glucagon (Crane *et al.*, 1982; Keppens and DeWulf, 1984).

ROLE OF PROTEIN KINASE C

Phorbol esters are a series of tumour promoting compounds which induce a great variety of effects in different cells and tissues. It has been shown that phorbol esters bind and stimulate the Ca^{2+} - activated phospholipid-dependent protein kinase, known as protein kinase C (Castagna *et al.*, 1982; Kikkawa *et al.*, 1983). The ability of phorbol esters, such as PMA, to activate protein kinase C (PK-C) directly, apparently by replacing the requirement for diacylglycerol, has provided a powerful tool to probe the actions of protein kinase C (PK-C) in the intact cell. Activation of this kinase seems to be the major route whereby these tumour promoters exert their actions.

Recently, it has become increasingly apparent that PK-C plays an important role in altering the cellular responsiveness to a variety of agents; this change in responsiveness seems to be associated with changes in the number, affinity or state of phosphorylation of various receptors and components of signal transduction systems (Houslay, 1991). The glucagon receptor was reported to be

phosphorylated, as activation of PK-C causes it to become uncoupled from G_s under conditions where it can be clearly shown that G_s does not become phosphorylated (Bushfield *et al.*, 1990). Dissociation between the cAMP concentrations and the metabolic effects induced by glucagon is evidenced in the presence of phorbol esters (Garcia-Sainz *et al.*, 1985). PMA diminished the accumulation of cAMP induced by glucagon, but had no significant effect on the stimulation of ureagenesis produced by glucagon.

In the studies using phorbol esters, as activators of protein kinase C, however it should be noted that the diacylglycerol produced in the cell may activate protein kinase C isoforms selectively, depending upon the fatty acid composition of the diacylglycerol and that such a spectrum and magnitude of activity may be very different from that achieved by phorbol esters (Houslay, 1991). Also, phorbol esters achieve a prolonged activation of protein kinase C and may cause its down-regulation (Nishizuka, 1988).

PROBLEMS OF INVESTIGATION

The research in this thesis can be divided into two main parts. The first part investigates the role of glucagon and glucagon-like peptides in the regulation of GCS, while the second part discusses the signalling pathway(s) of glucagon

stimulation of GCS.

Glucagon-related peptides such as GLP-1, oxyntomodulin and glicentin are becoming highly recognized for their sequence similarity with glucagon and their effects on the physiological regulation of many metabolic processes (Bloom and Polak, 1982; Holst et al., 1987). Therefore, the first part of this thesis deals with their effects on the flux through GCS and on glycogenolysis. We found that oxyntomodulin and glicentin stimulate the flux through the GCS and glycogenolysis. Therefore, the question arises as to whether glicentin and oxyntomodulin are exerting these effects by acting through the glucagon receptor. The glucagon receptor antagonists were used to examine this possibility.

As previously stated, GCS is known to be regulated by glucagon. The major unanswered question is how the hormonal signal is transmitted through the cytoplasm into the mitochondria and how does it affect mitochondrial enzymes such as GCS. In the second part of this thesis this problem was examined using various cell agonists and antagonists that affect certain parts of the signalling pathway.

Phosphorylation/ dephosphorylation processes play important roles in metabolic regulation. Therefore, The involvement of phosphorylated proteins in the regulation of GCS was examined by using the cytosolic protein phosphatase

inhibitor, okadaic acid. Okadaic acid was found to mimic hormonal actions on the flux through GCS which raised the question of possible involvement of phosphorylation/dephosphorylation processes in the glucagon stimulation of the GCS. This question was also examined in chapter 4.

Since protein kinase A is responsible for the phosphorylation of many cytosolic proteins, the possibility of cAMP-induced phosphorylation in the regulation of GCS was examined using the PK-A agonist Sp-cAMPS and the PK-A antagonist Rp-8-Br-cAMPS. The possible involvement of cAMP-dependent phosphorylation in the glucagon effect on GCS was also examined.

The fact that glucagon, like vasopressin and angiotensin II, is known to increase Ca^{2+} uptake in rat hepatocytes (Keppens et al., 1977) prompted us to investigate the role of calcium in the regulation of GCS. Vasopressin and angiotensin II are known to have no effect on adenylyl cyclase activity in the liver. In isolated hepatocytes, the effects of vasopressin and angiotensin II on the glycine cleavage system were examined. Phorbol esters have provided a powerful tool to investigate the actions of PK-C in the intact cells. In this study the phorbol ester PMA has been used to examine the question of whether PK-C is involved in the regulation of GCS and its effect upon the glucagon stimulation of GCS.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS

Chemicals

[1-¹⁴C] glycine and Omnifluor were obtained from DuPont New England Nuclear (Mississauga, Ontario). Collagenase, CLS2, was from Worthington Biochemical Corporation (New Jersey, USA). GLP-1 (7-36) amide, oxyntomodulin (glucagon 37), glucagon (19-29), and Des-His¹-Glu³-glucagon amide were from Bachem (California, USA). Sp- and Rp-8-Br-cAMPS were purchased from Biolog Life Science Institute, La Jolla, CA. Okadaic acid was obtained from Biomol Research Laboratories, Plymouth Meeting, PA. Glicentin was purchased from the American Peptide Company, Inc. Vasopressin and Angiotensin II, Phorbol 12-myristate 13-acetate and Phorbol 12-myristate 13-acetate 4-O-methyl ether were from Sigma (St. Louis, USA). Hexokinase and glucose-6-phosphate dehydrogenase were purchased from Boehringer Mannheim Biochemica (Laval, Quebec). Des-His¹-Nle⁵-Ala¹¹-Ala¹⁶-glucagon amide was a gift from Dr.C.G.Unson, The Rockefeller University, New York.

Animals

Male Sprague-Dawley rats (Charles River, Montreal) weighing 300-400 g were used for all studies. Rats were allowed water and purina rat chow ad libitum.

METHODS

Preparation of hepatocytes

Hepatocytes were prepared as described previously by Berry *et al.*, (1991). Rats were anaesthetized with pentobarbital 0.1 ml/ 100 gm body weight. The femoral vein was exposed and 0.2 ml of sodium heparin (1000 USP Units/ mL) was injected into it. The isolation of hepatocytes involved perfusing rat liver first with 500 ml of calcium-free Krebs-Henseleit bicarbonate medium (24 mM NaHCO_3 , 1.2 mM KH_2PO_4 , 1.2 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5 mM KCl, 123 mM NaCl) containing 2 mM EGTA, 20 mM glucose, 2.1 mM lactate, and 0.3 mM pyruvate, gassed with 95:5 $\text{O}_2:\text{CO}_2$. The flow rate in all cases was approximately 40 ml/ minute. This was followed by perfusing the liver with 500 ml of the same medium which contained 1.3 mM CaCl_2 and no EGTA. Calcium-containing Krebs-Henseleit medium containing lactate, pyruvate and glucose with 0.25% bovine serum albumin (Fraction V essentially fatty acid free) and 50 mg collagenase/ 100 ml was then recirculated for 15 to 20 minutes in a total volume of 100 ml.

When the liver started to leak it was removed and minced in a petri dish. The suspension was then shaken in a Dubnoff metabolic shaker at 37°C with gassing $\text{O}_2:\text{CO}_2$ (95:5) for 10 minutes. The resulting cells were spun down at 600 rpm for 2 minutes and washed twice more in Krebs-Henseleit medium. The

final wash was in Krebs-Henseleit medium containing 2.5% BSA and the final suspension of cells was also in this medium.

Determination of cell quantity

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 final resuspension medium, Krebs-Henseleit medium containing 2.5% BSA, was placed in a separate weighing pan. Both were dried in an oven at 50°C overnight. The difference in weight between the cells and the medium was used to determine the dry weight of the cells present.

Determination of cell viability

The viability of the isolated cells was determined by staining with 0.2% trypan blue in Krebs-Ringer phosphate. At least 200 cells were counted using a haemocytometer chamber and the percentage of cells stained with trypan blue was calculated. This organic amine dye is excluded from hepatocytes with intact membranes, whereas damaged cells readily take it up. Trypan blue is negatively charged and it seems likely that the dye is excluded as a result of an energy-dependent maintenance of a negative plasma membrane potential inside the hepatocytes. Loss of this potential, due

to cell injury, may allow trypan blue penetration (Berry *et al.*, 1991). In all experiments, the cell viability, as determined by trypan blue, was greater than 95%.

Preparation of mitochondria

Mitochondria were isolated from livers of male Sprague-Dawley rats as described previously by Jois *et al.*, (1989). Rats were killed by cervical dislocation and the liver was quickly removed. Then the liver was homogenized, using a Potter-Elvehjem 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 xg and the supernatant was then centrifuged at 8200 xg for 10 minutes. The resulting pellet was resuspended in the above medium and centrifuged at 8200 xg for 10 minutes. The resuspension and centrifugation steps were repeated two times and the final pellet was resuspended in the same 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 a standard.

Measurement of flux through GCS in isolated hepatocytes

Determining flux through GCS was done by measuring $^{14}\text{CO}_2$ production from 0.3 mM $[1-^{14}\text{C}]$ glycine (Hampson *et al.*, 1983; Jois *et al.*, 1989). This concentration of glycine was used as it approximates that in the hepatic portal blood (Brosnan *et al.*, 1983). Incubations were carried out at 37°C , in triplicate, in a total volume of 0.5 mL of Krebs-Henseleit medium containing between 4-6 mg dry weight of hepatocytes. Cells were preincubated in Krebs-Henseleit medium for 20 min before the addition of $[1-^{14}\text{C}]$ glycine. Each flask was gassed with 95:5 $\text{O}_2:\text{CO}_2$ for 20 seconds after the addition of the hepatocytes and also after the addition of $[1-^{14}\text{C}]$ glycine. For zero time samples 0.3 ml of 30% perchloric acid (w/v) was added before the hepatocytes. At the end of the incubation period 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. $^{14}\text{CO}_2$ was collected for 1 hour and the centre wells were then transferred to scintillation vials containing 15 ml of scintillation fluid (Omnifluor). Radioactivity was determined in a liquid scintillation

counter, with conversion to dpm made using an external standard.

GCS flux in isolated mitochondria

Isolated mitochondria were preincubated in 25 ml Erlenmeyer flasks for 20 minutes at 30°C before addition of [1-¹⁴C] glycine. All incubations were carried out in triplicate and each flask contained 2-3 mg mitochondrial protein. The incubation period was terminated with the addition of 0.3 ml of 30% perchloric acid (w/v). For zero time values the perchloric acid was added before the mitochondria. Centre wells containing NCS tissue solubilizer were suspended inside the incubation flasks to trap ¹⁴CO₂ released after termination of incubation with 30% perchloric acid. ¹⁴CO₂ was collected for 60 minutes. The centre wells were then transferred to scintillation vials containing 15 ml of scintillation fluid (Omnifluor) and counted.

Validity of using ¹⁴CO₂ release from [1-¹⁴C]glycine to determine the flux through GCS

Production of ¹⁴CO₂ from [1-¹⁴C] glycine by isolated mitochondria, isolated perfused liver and isolated hepatocytes has been shown to be predominantly due to flux through the GCS enzyme system (Hampson *et al.*, 1983; 1984; Jois *et al.*, 1989).

Hampson *et al.*, (1983) measured the production of $^{14}\text{CO}_2$ from $[1-^{14}\text{C}]$ - and $[2-^{14}\text{C}]$ glycine and the change in oxygen consumption upon the addition of glycine by mitochondria was also measured as a function of glycine concentration. The correlation between the measured mitochondrial oxygen consumption due to glycine oxidation and the calculated, or "theoretical," oxygen consumption based upon the production of $^{14}\text{CO}_2$ from $[1-^{14}\text{C}]$ - and $[2-^{14}\text{C}]$ glycine proved that GCS is the predominant fate of the added glycine. They also demonstrated that addition of 1 mM sodium arsenite and 1 mM aminooxyacetic acid significantly inhibited the production of $^{14}\text{CO}_2$ from $[1-^{14}\text{C}]$ glycine. Arsenite poison thiols and therefore inhibits the lipoamide-dependent reaction of glycine cleavage. Aminooxyacetic acid is a pyridoxal phosphate antagonist and therefore, it would inhibit the GCS. These experiments indicated that the metabolic flux through GCS can be monitored effectively, in an intact and fully functional mitochondrial system, by measuring the production of $^{14}\text{CO}_2$ from $[1-^{14}\text{C}]$ glycine. In perfused rat liver the production of $^{14}\text{CO}_2$ from $[1-^{14}\text{C}]$ - and $[2-^{14}\text{C}]$ glycine was examined (Hampson *et al.*, 1984). At a glycine concentration of 10 mM, production of $^{14}\text{CO}_2$ from $[1-^{14}\text{C}]$ glycine was maximal, exhibiting a maximal activity of 125 nmol of $^{14}\text{CO}_2$ / g/ min, compared to production of $^{14}\text{CO}_2$ from $[2-^{14}\text{C}]$ glycine which exhibited a maximal activity of 40 nmol

of $^{14}\text{CO}_2/\text{g}/\text{min}$. Washout kinetic experiments, in perfused rat liver, with $[1-^{14}\text{C}]$ glycine exhibited a single half-time of $^{14}\text{CO}_2$ disappearance, indicating one metabolic pool from which $^{14}\text{CO}_2$ is derived (Hampson *et al.*, 1984). In isolated hepatocytes Jois *et al.*, (1989) demonstrated that the production of $^{14}\text{CO}_2$ from $[1-^{14}\text{C}]$ glycine was much higher than that from $[2-^{14}\text{C}]$ glycine and that the GCS is the major route for glycine decarboxylation.

Glycogenolysis in isolated hepatocytes

Glycogenolysis was determined in hepatocytes isolated from fed rats as the glucose produced over the incubation period. Incubations were done in triplicate with a final volume of 1 ml in 25 ml Erlenmeyer flasks. Typical incubations contained 4-6 mg dry wt of cells. The incubations were carried out in Krebs-Henseleit medium, gassed with 95:5 $\text{O}_2:\text{CO}_2$ at 37°C in a Dubnoff metabolic shaker. Before the incubation period the cells were preincubated for 20 minutes. The glucose formed during this preincubation period was subtracted from the glucose formed during the 30 minute incubation period. After the 30 minutes incubation period 0.5 ml aliquots were removed from the flasks into Eppendorf tubes containing 50 μl of 30% perchloric acid and centrifuged for 2 minutes at $12,000 \times g$. The supernatant was neutralized with 3 M potassium phosphate

(K_2PO_4) and the glucose was assayed in the supernatant using a standard enzymatic glucose-6-phosphate dehydrogenase/hexokinase method (Bergmeyer *et al.*, 1974).

Attempts to increase the sensitivity of the hepatocytes

At the begining of this research I examined a number of strategies that were expected to increase the sensitivity of isolated hepatocytes to hormonal stimulation. These experiments included: 1) Percoll treatment of hepatocytes. 2) Reduction of the duration of incubations. 3) Reduction of the concentration of isolated hepatocytes in the final incubation medium.

1-Removal of damaged hepatocytes by treatment with Percoll

Percoll is a colloidal solutions which consists of polyvinyl pyrrolidine-coated colloidal silica-gel particles (Pertoft *et al.*, 1977; 1979). It forms an iso-osmotic gradient within a density range of 1-1.3 gm/ml. The great advantage of Percoll compared with other gradient materials, such as Metrizamide and Sodium Metrizoate, is its low contribution to osmotic pressure (Pertoft *et al.*, 1977). High density together with iso-osmolality, physiological ionic strength and high survival rate of rat liver cells could only be obtained in Percoll solutions. Pertoft *et al.*, (1977) reported that

Percoll does not impair the growth and the survival of the rat liver cells. Smedsrod and Pertoft, (1985) demonstrated that Percoll centrifugation is a rapid method for mass isolation of functionally intact hepatocytes and reticuloendothelial cells from rat liver. Ultrastructure analysis shows that Percoll does not connect with the cells during the separation procedure. The use of Percoll as the gradient medium allows gradient formation and cell separation to take place simultaneously.

First a Percoll isotonic solution was prepared from Percoll, balanced salt solution and phosphate buffer and the pH was adjusted to 7.4 with 0.1 M HCl. The balanced salt solution was prepared by dissolving 80g NaCl, 4g KCl and 2g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in 1l H_2O . A phosphate buffer was prepared comprising 2.4g Na_2HPO_4 and 0.4g KH_2PO_4 , dissolved in 200 ml H_2O . Equal volumes of cell suspension and Percoll solution were added to 50 ml centrifuge tubes. They were mixed gently and centrifuged at 0-4°C at 50 xg for 10 minutes. Cells capable of excluding trypan blue will pellet, whereas damaged cells, non-parenchymal cells, cell aggregates and debris will float in the upper region of each tube. To remove the Percoll, the supernatant was aspirated and the pelleted cells were resuspended in the washing medium and centrifuged for 3 minutes at 40 xg. The washing step was repeated and the pelleted cells were resuspended in Krebs-Henseleit medium

containing 2.5% BSA (Berry *et al.*, 1991).

The advantage of such a technique is that it only employs low speed centrifugation since high speed centrifugation involving use of a Percoll gradient has been demonstrated to affect the sensitivity of isolated hepatocytes. It has been reported that high speed centrifugation of isolated hepatocytes through a Percoll gradient impaired the ability of insulin to stimulate both the oxidation of labelled succinate to CO₂ and the incorporation of carbon atoms from this compound into protein (Berry *et al.*, 1991).

2- Reduction of the duration of incubations

The 20 minute preincubation period was eliminated and the hepatocytes were incubated directly with glucagon and 1-¹⁴C glycine for 30 minutes. The incubation was terminated and ¹⁴CO₂ was collected as described earlier. The sensitivity of the hepatocytes to glucagon stimulation was compared with the sensitivity of hepatocytes that were preincubated for 20 minutes before being challenged with the glucagon. The stimulation of the flux through GCS and glycogenolysis, as a function of glucagon concentration, were taken as a measure of the sensitivity of the hepatocytes. The two sets of hepatocytes were from the same rat liver and measurement of the effect of glucagon on GCS flux and glycogenolysis was done

simultaneously, under the same experimental conditions.

3- Reduction of the concentration of the hepatocytes

After isolation of the hepatocytes they are regularly suspended 1:18 in Krebs-Henseleit medium containing 2.5% BSA. In order to determine whether concentration of cells affects their response, an aliquot of hepatocytes was diluted 10 times further (to 1:180) in the same medium. Their sensitivity to stimulation by glucagon, assessed as stimulation of GCS flux and glycogenolysis, was measured and compared to that of hepatocytes which were more concentrated (1:18). Both sets of hepatocytes were isolated from the same rat and were preincubated for 20 minutes then incubated for 30 minutes with the glucagon and [$1\text{-}^{14}\text{C}$] glycine simultaneously and under the same conditions.

Statistical analysis

All values were expressed as mean of three separate experiments \pm SD. Statistical analysis was done by Student's t test. Multiple comparisons were tested using ANOVA and the Tukey multiple comparison test. A probability, $p < 0.05$, was regarded as indicating statistical significance.

Maximal stimulation and half maximal stimulation were calculated using the computer program Inplot which uses the equation for a rectangular hyperbola

$$Y = A * X / (B + X)$$

This equation describes the binding of a ligand to a receptor as a function of its concentration. It is also known as a saturation binding curve or a binding isotherm. Y is the stimulation of GCS which increases to a plateau value of A (maximal stimulation). B is the dissociation constant (half maximal stimulation). Maximal stimulation (A) is expressed as nmoles/ 30 min/ mg dry wt ; Half maximal stimulation (B) is expressed as Molar concentration. In some experiments in which very high glucagon concentrations began to inhibit the GCS flux only those concentrations which were stimulatory were used. It is recognized that the phenomena observed in this work are more complex than simple ligand binding but are well described by this equation as indicated by r^2 values of more than 0.95.

RESULTS AND DISCUSSION

CHAPTER 3

INTRODUCTION

The GCS enzyme system is known to be regulated by glucogenic hormones such as glucagon (Jois *et al.*, 1989; Brosnan *et al.*, 1990). Glucagon-related peptides such as oxyntomodulin, glicentin and GLP-1 (7-36) amide share sequence similarity with glucagon and also play important regulatory roles. Therefore, we decided to study their effect on the flux through GCS. The effect of glucagon (19-29) on the flux through GCS was also examined. In addition, glucagon receptor antagonists were used to determine whether the glucagon-related peptides were able to affect GCS flux and glycogenolysis via interaction with the glucagon receptor.

ATTEMPTS TO INCREASE THE SENSITIVITY OF ISOLATED HEPATOCYTES

Glucagon stimulates the flux through GCS and glycogenolysis in isolated rat hepatocytes with a half maximal stimulation which occurs at glucagon concentrations of 4.3 ± 1.3 nM and 3.3 ± 0.8 nM, respectively (Fig. 3.1). Three different approaches were examined at the beginning of this research project in an effort to increase the sensitivity of the isolated hepatocytes to glucagon stimulation. These experiments included:

- 1) Percoll treatment of isolated hepatocytes.
- 2) Reducing

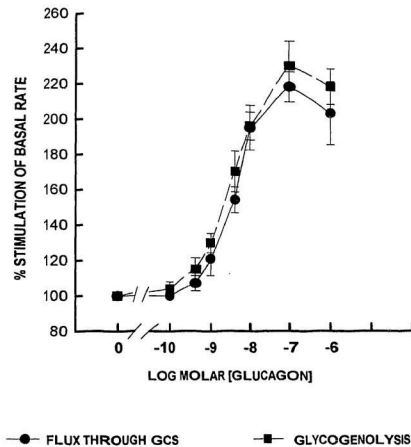
Fig. 3.1 Stimulation of the flux through GCS and glycogenolysis by glucagon in isolated rat hepatocytes

The basal rate for GCS flux was 1.3 ± 0.9 nmoles/ 10 min/ mg dry wt. and for glycogenolysis was 176 ± 33 nmoles/ 10 min/ mg dry wt. Results are expressed as the percentage of the unstimulated rate for both GCS flux and glycogenolysis. Results are means of three separate experiments \pm SD.

Fig. 3.1

GLUCAGON STIMULATION OF THE FLUX THROUGH GCS

AND GLYCOGENOLYSIS



the duration of incubations. 3) Decreasing the concentration of the hepatocytes in the final incubation volume.

Percoll treatment of isolated hepatocytes

Glucagon stimulation of the flux through GCS and glycogenolysis was measured in isolated hepatocytes prepared regularly and in Percoll purified isolated hepatocytes. No significant difference was detected in the half maximal stimulation or in the maximal stimulation of $^{14}\text{CO}_2$ or glucose production as shown in table 3.1.

Reduction of the duration of incubation

The sensitivity of isolated hepatocytes that were preincubated for 20 minutes before the incubation was compared to another group without the preincubation period. No significant difference was found in half maximal stimulation or maximal stimulation of GCS flux or glycogenolysis by glucagon between both groups (table 3.2).

Decreasing the concentration of hepatocytes in the final incubation volume

Isolated hepatocytes that were regularly suspended 1:18 in Krebs-Henseleit medium containing 2.5% BSA did not show any significant difference in their response to glucagon stimulation, as measured by the flux through GCS and

Table 3.1 Effect of Percoll purification of isolated hepatocytes on their sensitivity to glucagon stimulation of the flux through GCS and glycogenolysis

	Flux through GCS		Glycogenolysis	
	Half maximal stimulation (nM)	Maximum GCS flux rate (nmoles/30min /mg dry wt.)	Half maximal stimulation (nM)	Maximum glucose production rate (nmoles/30min /mg dry wt.)
With Percoll	3.6 ± 0.4	3.9 ± 0.9	3.8 ± 0.2	335 ± 44
Without Percoll	3.3 ± 0.7	3.9 ± 1.1	3.3 ± 1.0	320 ± 32

Both GCS and glycogenolysis were measured. Both groups were compared using paired t-test, $p > 0.05$. Basal rate of GCS flux is 1.52 ± 0.89 nmoles/ 30min/ mg dry wt. Basal rate of glycogenolysis is 184.4 ± 22 nmoles/ 30 min/ mg dry wt. Results are means from three separate experiments \pm SD.

Table 3.2 Effect of preincubation of hepatocytes on their sensitivity to glucagon stimulation of the flux through GCS and glycogenolysis

	Flux through GCS		Glycogenolysis	
	Half maximal stimulation (nM)	Maximum GCS flux rate (nmoles/30min /mg dry wt.)	Half maximal stimulation (nM)	Maximum glucose production rate (nmoles/30min /mg dry wt.)
Preincubation	3.3 ± 0.6	3.3 ± 1.0	3.6 ± 0.8	398 ± 59
No preincubation	3.2 ± 0.9	3.2 ± 0.3	3.3 ± 0.6	387 ± 47

Basal rate for GCS is 1.7 ± 0.9 and for glucose production is 198 ± 40 nmoles/ 30 min/ mg dry wt. Both groups were compared using paired t-test, $p > 0.05$. Results are means from three separate experiments \pm SD.

glycogenolysis, when compared to isolated hepatocytes that were suspended 1:180 in the same medium. Half maximal stimulation and maximal stimulation of GCS flux and glycogenolysis did not differ significantly (Table 3.3).

Since we were unable to increase the sensitivity of isolated hepatocytes to glucagon by these means we proceeded with our experiments with regularly isolated hepatocytes, preincubated for 20 minutes and then incubated for 30 minutes at a concentration of about 8-12 mg cells/ ml.

EFFECT OF GLUCAGON-RELATED PEPTIDES ON GCS FLUX

The first question that was examined was whether glucagon-related peptides such as oxyntomodulin, glicentin and GLP-1 (7-36) amide stimulate the flux through GCS in the isolated hepatocytes. When incubated with isolated hepatocytes, oxyntomodulin and glicentin stimulated the flux through GCS at high concentration of 1 μ M and 0.1 μ M, respectively (Fig. 3.2). The maximum stimulation obtained with oxyntomodulin was about 100% at a concentration of 10 μ M of the peptide. Meanwhile, the maximum stimulation obtained with glicentin was about 60% at a concentration of 1 μ M glicentin. Similar results were obtained with glycogenolysis for both oxyntomodulin and glicentin (Fig. 3.3).

Table 3.3 Effect of hepatocyte concentration on their sensitivity to glucagon stimulation of GCS flux and glycogenolysis

	Flux through GCS		Glycogenolysis	
	Half maximal stimulation (nM)	Maximum GCS flux rate (nmoles/30min /mg dry wt.)	Half maximal stimulation (nM)	Maximum glucose production rate (nmoles/30min /mg dry wt.)
1:18 dilution	5.2 ± 1.9	2.1 ± 0.7	5.3 ± 0.9	279 ± 23
1:180 dilution	5.3 ± 0.9	2.1 ± 0.8	4.4 ± 1.3	290 ± 31

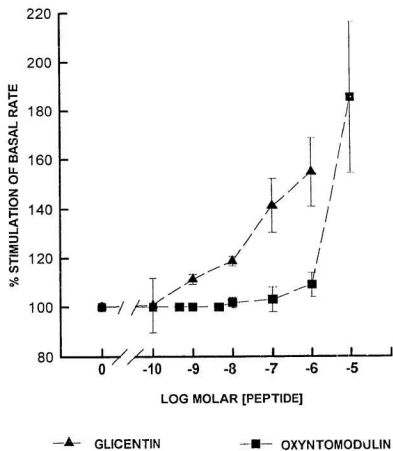
Basal rate of GCS flux is 1.02 ± 0.93 nmoles/ 30 min/ mg dry wt. Basal rate of glycogenolysis is 156 ± 34.8 nmoles/30 min/ mg dry wt. Results are the means of three separate experiments. Student's t-test was used to compare half maximal stimulation and maximal rate of GCS and glucose production for both groups. No significant difference was detected, $p > 0.05$.

**Figure 3.2 Stimulation of the flux through GCS by
oxyntomodulin and glicentin in isolated rat hepatocytes**

Different preparations of isolated hepatocytes were preincubated for 20 minutes before they were incubated with 0.3 mM 1-¹⁴C glycine and oxyntomodulin or glicentin for 30 minutes. Results are expressed as the percentage of the unstimulated rate. The basal rates for GCS flux are 1.2 ± 0.1 and 2.4 ± 0.6 nmoles/ 30 min/ mg dry wt. for oxyntomodulin and glicentin, respectively. Results are the means of three different experiments \pm SD.

Fig. 3.2

STIMULATION OF FLUX THROUGH GCS IN ISOLATED
RAT HEPATOCYTES

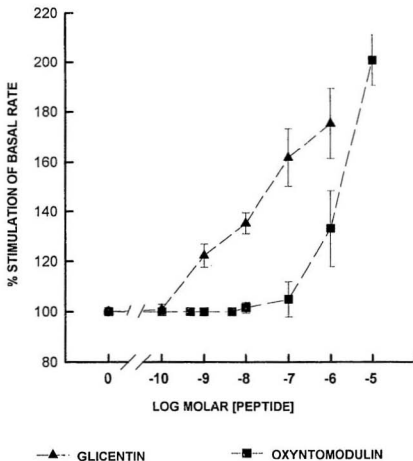


**Figure 3.3 Oxyntomodulin and glicentin stimulation of
glycogenolysis in isolated rat hepatocytes**

Hepatocytes were preincubated for 20 minutes before the addition of oxyntomodulin or glicentin. The basal rates were 105 ± 10 and 183 ± 13 nmoles glucose/ 30 min/ mg dry wt. for oxyntomodulin and glicentin, respectively. Results are means \pm SD; n = 3.

Fig. 3.3

STIMULATION OF GLYCOGENOLYSIS IN ISOLATED
RAT HEPATOCYTES



GLP-1 (7-36) amide was found to be without effect on the GCS or glycogenolysis in isolated rat hepatocytes as shown in table 3.4. The peptide was without effect on the unstimulated basal rate of the release of $^{14}\text{CO}_2$ or glucose production as well. When preincubated with isolated hepatocytes before they were challenged with glucagon, it was found that GLP-1 (7-36) amide had no effect on the glucagon stimulation of the flux through GCS (Fig. 3.4) or glycogenolysis (Fig. 3.5).

The question of a possible role of glucagon (19-29), which is also known as miniglucagon, on the metabolic processes in the liver was investigated by examining its effect on the flux through GCS and glycogenolysis. Incubation of isolated hepatocytes with miniglucagon had no effect on the flux through GCS or glycogenolysis (Fig. 3.6).

Is the stimulation of GCS by glicentin and oxyntomodulin brought about via interaction with the glucagon receptor?

Glucagon receptor antagonists were used to examine this question. Another question which could be considered here is whether hepatocytes possess two distinct receptors for glucagon or just one receptor that leads to two different signals. In all the experiments that were done with receptor antagonists glycogenolysis was measured so as to compare their

Table 3.4 Effect of GLP-1 (7-36) amide on the flux through GCS and glycogenolysis in isolated rat hepatocytes

	Flux through GCS (nmoles/30min/mg dry wt.)	Glycogenolysis (nmoles/30min/mg dry wt.)
Basal rate	2.07 ± 0.68	187 ± 35
10⁻⁶M GLP-1	2.11 ± 0.98	196 ± 45

Isolated hepatocytes were preincubated with GLP-1 (7-36) amide for 20 minutes before the reaction was initiated by the addition of 1-¹⁴C glycine. ¹⁴CO₂ was collected and counted and glycogenolysis was measured. Results are means ± SD, n=3. Both groups were compared using paired t-test, p > 0.05.

Figure 3.4 Effect of GLP-1 (7-36) amide on glucagon-stimulated flux through GCS in isolated hepatocytes

Isolated hepatocytes were preincubated for 20 minutes without (●) and with (■) GLP-1 (7-36) amide before they were challenged with glucagon. Half maximal stimulation of the flux through GCS occurred at (●) 3.1 ± 0.9 vs. (■) 3.4 ± 0.4 nM glucagon concentration ($p > 0.05$, Paired t-test). Results are means of four different experiments \pm SD.

Fig. 3.4

EFFECT OF GLP-1 ON GLUCAGON-STIMULATED GCS
FLUX IN ISOLATED RAT HEPATOCYTES

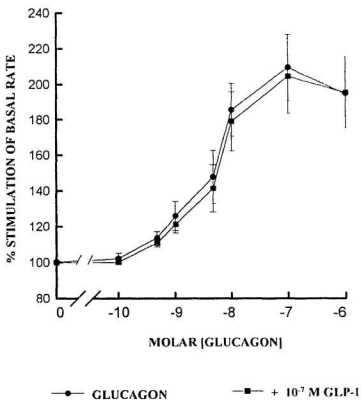


Figure 3.5 Effect of GLP-1 (7-36) amide on glucagon - stimulated glycogenolysis in isolated hepatocytes

A glucagon dose response curve for glycogenolysis was done (■) with and without (●) the presence of 100 nM GLP-1 (7-36) amide. Half maximal stimulation of the glucagon curve occurred at (■) 2.3 ± 0.6 nM vs. (●) 2.6 ± 0.5 nM glucagon concentration ($p > 0.05$, paired t-test). Results are expressed as the percentage of the unstimulated basal rate. Results are the means of four different experiments \pm SD.

Fig. 3.5

EFFECT OF GLP-1 ON GLUCAGON-STIMULATED
GLYCOGENOLYSIS IN ISOLATED RAT HEPATOCYTES

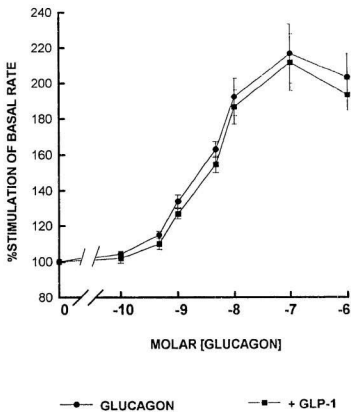
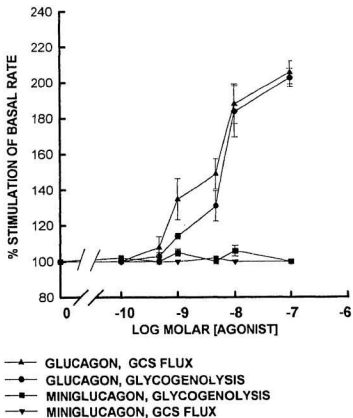


Fig. 3.6 Effect of miniglucagon on the flux through GCS and glycogenolysis in isolated rat hepatocytes

Isolated hepatocytes were preincubated for 20 minutes, then they were incubated with miniglucagon and 0.3 mM 1-¹⁴C glycine for 30 minutes. Results are expressed as the percentage of the unstimulated basal rate. The basal rates are 1.5 ± 0.6 nmoles/30 min/ mg dry wt. for GCS flux and 107 ± 15 nmoles glucose/30 min/ mg dry wt. for glycogenolysis. $n = 3$.

Fig. 3.6

**EFFECT OF MINIGLUCAGON ON THE FLUX THROUGH GCS AND
GLYCOGENOLYSIS IN ISOLATED RAT HEPATOCYTES**



effects on the glucagon-induced stimulation of glycogenolysis (representing a cytoplasmic process) and the flux through GCS (representing a mitochondrial process).

Preincubation of the hepatocytes with various concentrations of the glucagon receptor-antagonist des-His¹-Glu⁹-glucagon amide was done before hepatocytes were challenged with glucagon, glicentin, or oxyntomodulin. A significant inhibition of about 40% of the stimulatory effect of glucagon (Fig. 3.7), oxyntomodulin (Fig. 3.8) and glicentin (Fig. 3.9) on the flux through GCS and glycogenolysis was found. It was not possible to attempt higher concentrations of this antagonist due to its expense.

Fig. 3.10 shows that the stimulatory effect of glucagon on the flux through GCS and glycogenolysis was inhibited by 80% when the hepatocytes were preincubated with 10 μ M des-His¹-Nle⁹-Ala¹¹-Ala¹⁶-glucagon amide, before they were challenged with glucagon.

Fig. 3.7 Inhibition of glucagon-stimulated flux through GCS and glycogenolysis by des-His¹-Glu²-glucagon amide

Flux through GCS and glycogenolysis were determined in isolated rat hepatocytes. The antagonist was preincubated with the hepatocytes for 20 minutes before they were challenged with 10 nM glucagon. ¹⁴CO₂ and glucose production during the 30 minutes incubation period was determined. Control incubations were made with the antagonist alone to determine if it had an effect on the basal rate of GCS flux or glucose production. The basal rate for GCS flux is 1.4 ± 0.5 nmoles/ 30 min/ mg dry wt. and for glucose production is 95 ± 8.6 nmoles/ 30 min/ mg dry wt. (*p < 0.05 vs. control with no antagonist). Results are means \pm SD, n = 3.

Fig. 3.7

INHIBITION OF GLUCAGON STIMULATED GCS FLUX AND
GLYCOGENOLYSIS BY DES-HIS¹-GLU⁹-GLUCAGON AMIDE

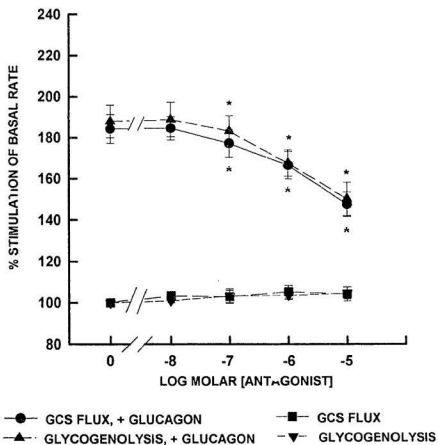


Fig. 3.8 Inhibition of oxyntomodulin stimulated flux through GCS and glycogenolysis by des-His¹-Glu⁹-glucagon amide in isolate hepatocytes

Isolated rat hepatocytes were preincubated with the antagonist for 20 minutes before the reaction was initiated by the addition of 10^{-5} M oxyntomodulin and 0.3 mM 1-¹⁴C glycine. Flux through GCS and glycogenolysis were measured. Control incubations with the antagonist alone for both GCS flux and glycogenolysis were carried out. The basal rate for the flux through GCS is 1.9 ± 0.2 nmoles/ 30 min/ mg dry wt. The basal rate for glucose production is 108 ± 10 nmoles/ 30 min/ mg dry wt. (* $p < 0.05$ vs. control with no antagonist). Results are means \pm SD of three separate experiments.

Fig. 3.8

INHIBITION OF OXYNTOMODULIN STIMULATED GCS FLUX
AND GLYCOGENOLYSIS BY DES-HIS¹-GLU⁹ GLUCAGON AMIDE

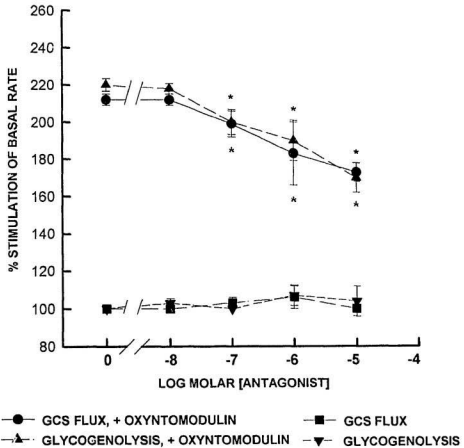


Fig. 3.9 Inhibition of glicentin stimulation of the flux through GCS and glycogenolysis by des-His¹-Glu⁹-glucagon amide

Des-His¹-Glu⁹-glucagon amide was preincubated with the isolated cells at 37°C before the addition of 1-¹⁴C glycine and 10⁻⁶ M glicentin at the beginning of the incubation period. Results are expressed as percentage of the unstimulated basal rate. The basal rates are 1.4 ± 0.1 and 136 ± 38 nmoles/ 30 min/ mg dry wt. for GCS flux and glycogenolysis, respectively. Results are the means of three separate experiments ± SD. * p < 0.05 vs. control with no antagonist; by ANOVA.

Fig. 3.9

INHIBITION OF GLICENTIN STIMULATED GCS FLUX AND
GLYCOGENOLYSIS BY DES-HIS¹-GLU⁹-GLUCAGON AMIDE

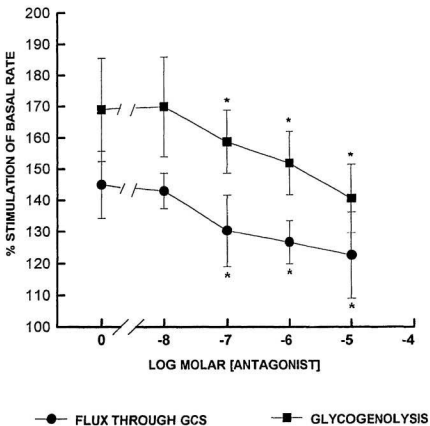
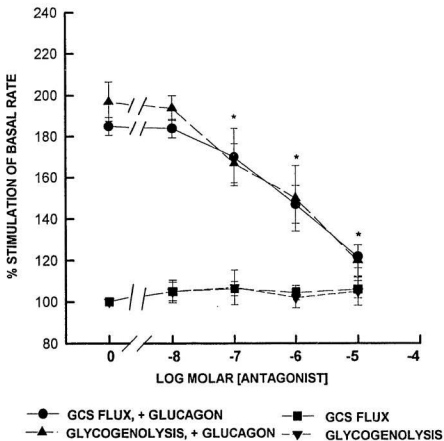


Fig. 3.10 Inhibition of glucagon stimulation of the flux through GCS and glycogenolysis by des-His¹-Nle²-Ala³-Ala⁴ glucagon amide

¹⁴CO₂ and glucose production was measured to determine the flux through GCS and glycogenolysis in isolated hepatocytes. The antagonist was preincubated with the cells for 20 minutes before adding glucagon and 1-¹⁴C glycine. GCS flux and glycogenolysis were measured as nmoles of ¹⁴CO₂ or glucose produced/ 30 min/ mg dry wt and were expressed as percentage of the unstimulated rate. The basal rates are 2.6 ± 0.8 nmoles/ 30 min/ mg dry wt. for GCS flux and 114 ± 9.6 nmoles/ 30 min/ mg dry wt. for glycogenolysis. Results are means ± SD, n = 3. *p < 0.05 vs. control with no added antagonist, by ANOVA.

Fig. 3.10
INHIBITION OF GLUCAGON STIMULATED GCS FLUX AND
GLYCOGENOLYSIS BY DES-HIS¹-NLE⁹-ALA¹¹-ALA¹⁶-GLUCAGON AMIDE



DISCUSSION

The sensitivity of hepatocytes to glucagon are different in different experimental systems. The sensitivity of isolated hepatocytes in batch incubation to glucagon stimulation has been reported to be less than that of perfused hepatocytes or the perfused liver at the same concentration of glucagon as measured by stimulation of glycogenolysis (Mine *et al.*, 1990). Half maximal stimulation of glycogenolysis in isolated rat hepatocytes occurred at 10^{-9} M glucagon compared to 9×10^{-11} M and 5×10^{-11} M in perfused hepatocytes and perfused liver, respectively. Several mechanisms have been suggested for that difference in the sensitivity to glucagon stimulation. The first possibility is that hepatocytes are partially damaged during the isolation so they only respond to high concentrations of glucagon. However, the sensitivity of the same isolated hepatocytes to glucagon increases when cells are stimulated in flow-through perfusion system. The second possibility is that glucagon is degraded in the batch incubation system; however, addition of agents that inhibit degradation of glucagon to the batch incubation medium concomitantly with glucagon had no effect on the glucose output in response to glucagon (Mine *et al.*, 1990). They suggested the existence of an inhibitory substance(s) secreted from glucagon-stimulated hepatocytes which may account for the

insensitivity of hepatocytes to glucagon observed in a batch incubation system.

The three different experimental approaches which were examined in this thesis were not able to increase the sensitivity of the isolated hepatocytes to glucagon stimulation. In our system, glucagon stimulates the flux through GCS and glycogenolysis with a half maximal stimulation at 4.35 ± 1.32 nM and 3.38 ± 0.83 nM glucagon, respectively. This is similar to that obtained by Mine *et al.*, (1990) and Corvera *et al.*, (1984). We find that the sensitivity cannot be readily increased in any of the ways described earlier.

In the present study, the effect of glucagon-related peptides, oxyntomodulin, glicentin, and GLP-1 on the flux through GCS and glycogenolysis were examined. Results shown in Fig. 3.2 and Fig. 3.3 clearly show that the flux through GCS and glycogenolysis are stimulated by oxyntomodulin and glicentin. This is consistent with the results reported by Kervran *et al.*, (1990) who found that, *in vivo*, oxyntomodulin displays a hyperglycemic effect at a dose 10 times higher than the dose of glucagon required to produce the same effect. *In vitro*, in isolated hepatocytes oxyntomodulin stimulated glycogenolysis but required a much higher dose than did glucagon (Kojima *et al.*, 1988). Although the concentrations of oxyntomodulin and glicentin that were used in our experiments

were very high, the involvement of these peptides in regulating the flux through GCS and glycogenolysis might occur in pathological cases.

Table 3.4 shows no effect of GLP-1 (7-36) amide on the flux through GCS or on glycogenolysis. This is consistent with the results of Blackmore *et al.*, (1991) They showed that GLP-1 (7-37), which has a similar action to GLP-1 (7-36) amide, does not stimulate hepatic glycogenolysis (as measured by phosphorylase activation) or gluconeogenesis (measured by the conversion of ^{14}C -lactate to ^{14}C -glucose) in rats. Fig. 3.4 and Fig. 3.5 also show no effect of GLP-1 (7-36) amide on the glucagon stimulation of GCS flux and glycogenolysis. Specific binding sites for the amidated peptides, which do not show binding to insulin or glucagon, in hepatocyte plasma membranes was reported by Villanueva-Pencarrillo *et al.*, (1993). In addition, it has been reported that GLP-1 (7-37) does not displace glucagon from its receptor(s) on the hepatic plasma membrane (Ghiglionne *et al.*, 1985). These results are consistent with the absence of effect of GLP-1 on glucagon stimulation of GCS and glycogenolysis. Valverde *et al.*, (1994) reported that 10^{-8}M GLP-1 reduced the cAMP content of glucagon-stimulated hepatocytes only in the absence of 3-isobutyl-1-methylxanthine (IBMX), a nonspecific cAMP phosphodiesterase inhibitor, which indicates that GLP-1 may stimulate a cAMP

phosphodiesterase activity. However, as will be discussed in chapter 4, PKA and cAMP are not the only factors involved in the regulation of GCS by glucagon.

Fig. 3.6 shows no effect of miniglucagon on the flux through GCS and glycogenolysis at any concentration tested. Mallat *et al.*, (1987) reported that both miniglucagon and glucagon (18-29) have no effect on hepatic adenylyl cyclase activity. However our reason for examining miniglucagon lay in its possible inhibition of the calcium pump with a possible increase in intracellular calcium. Without measurements of intracellular calcium, we can make no further comments on the lack of effect of miniglucagon.

Des-His¹-Glu⁹-glucagon amide which is known to be a potent antagonist of the glucagon receptor inhibited the stimulation of flux through GCS and glycogenolysis by glucagon, oxyntomodulin and glicentin. These results suggest that oxyntomodulin and glicentin are exerting their effects through their binding to glucagon receptor(s) in liver plasma membrane.

It has been suggested that hepatocytes possess two distinct receptors for glucagon: a GR-1 receptor coupled to stimulation of inositol phospholipid breakdown and a GR-2 receptor coupled to stimulation of adenylate cyclase activity (Wakelam *et al.*, 1986). It is conceivable, therefore, that

glycogenolysis and glycine catabolism could be stimulated via separate glucagon receptors. If this were the case one would expect them to display differential sensitivity to glucagon receptor antagonists. The results shown in Fig. 3.7 show that this was not the case since GCS flux and glycogenolysis were equally inhibited at the same concentration of the antagonist. These results were confirmed again by using a more potent glucagon receptor antagonist des-His¹-Nle⁶-Ala¹¹-Ala¹⁶-glucagon amide as shown in Fig. 3.10. These results cannot answer our question, however, because there are two possible explanations. The first possibility is the presence of two different glucagon receptors to which the glucagon receptor antagonists bind with the same affinity. The second possible explanation would be that there is only one receptor but two different G proteins, and it is their relative activation by the hormone-receptor complex that is different and is responsible for the difference in the release of the two messengers in response to the analogue. This is consistent with the results reported by Jelinek et al., (1993) who isolated a complementary DNA clone for the glucagon receptor and proved that it binds glucagon and transduces signals that leads to the accumulation of both cAMP and Ca²⁺.

CHAPTER 4

INTRODUCTION

Although the stimulation of GCS flux by glucagon has been demonstrated previously, it is not known how the hormonal signal is transmitted from the glucagon receptors on liver plasma membrane through the cytoplasm to the mitochondria. This problem was examined using different agents that affect different parts of the signalling pathway.

Protein phosphorylation and flux through GCS

The possibility of involvement of phosphorylated proteins was examined using okadaic acid which is a potent cell-permeable inhibitor of the cytoplasmic protein phosphatases PP1 and PP2A (Cohen and Cohen, 1989). Okadaic acid stimulated the flux through GCS and glycogenolysis in isolated hepatocytes (Fig. 4.1). The maximal effect was found at 1 μ M okadaic acid. A control experiment showed that okadaic acid had no direct effect on GCS in isolated mitochondria (Table 4.1). This suggests that the stimulation found in isolated hepatocytes is due to the well established inhibition of cytosolic protein phosphatases and increase in the concentration of phosphorylated proteins, rather than a direct effect at the level of the mitochondria.

A glucagon dose-response curve for the flux through GCS was done in the presence of a submaximal concentration of

Fig. 4.1 Effect of okadaic acid on the flux through GCS and glycogenolysis

Okadaic acid was added to the hepatocytes at the start of a 20 minute preincubation at 37°C, after which they were incubated with 0.3 mM 1-¹⁴C glycine for 30 minutes. The flux through GCS was measured by the nmoles of ¹⁴CO₂ released/ 30 min/ mg dry wt. Before the reaction was terminated aliquots were removed from the incubation flasks to determine the amount of glucose produced as nmoles/ 30 min/ mg dry wt. The basal rate for GCS flux is 1.1 ± 0.3 nmoles/ 30 min/ mg dry wt. and for glycogenolysis is 235 ± 38 nmoles/ 30 min/ mg dry wt. Results are means \pm SD; n = 3.

Fig 4.1

DOSE-RESPONSE CURVE WITH OKADAIC ACID

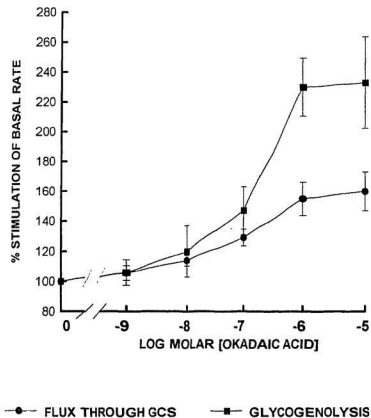


Table 4.1 Effect of okadaic acid on the flux through GCS in isolated mitochondria

	Flux through GCS (nmoles $^{14}\text{CO}_2$ released/ mg mitochondrial protein/min.)
Control, DMSO	0.16 \pm 0.02
okadaic acid, 1 μM	0.17 \pm 0.03

Isolated mitochondria were preincubated at 30°C for 20 minutes with 1 μM okadaic acid and DMSO as a control since okadaic acid is prepared in DMSO. Then they were incubated with 0.3 mM 1- ^{14}C glycine for 20 minutes and the reaction was stopped with the addition of 0.3 ml of 30% PCA and $^{14}\text{CO}_2$ was collected for an hour. The amount of $^{14}\text{CO}_2$ released was used to determine the flux through GCS, calculated as nmoles of $^{14}\text{CO}_2$ / mg mitochondrial protein /min. Results are means of three separate experiments \pm SD.

okadaic acid ($0.01 \mu\text{M}$). This concentration of okadaic acid was expected to produce a left shift in the glucagon dose-response curve, but it did not. The half maximal stimulation occurred at 3.5 ± 1.3 vs. 2.0 ± 0.1 nM ($p > 0.05$ in absence or presence of okadaic acid) as shown in Fig. 4.2. Similar results were obtained when these experiments were repeated for the glucagon stimulation of glycogenolysis. Half maximal stimulation occurred at 2.5 ± 0.2 vs. 2.3 ± 1.6 nM in the presence of $0.01 \mu\text{M}$ okadaic acid, as shown in Fig. 4.3. When hepatocytes were preincubated with a high concentration of okadaic acid (10^{-6}M) before they were challenged with glucagon, a significant left shift of the glucagon dose-response curve for GCS and glycogenolysis occurred (Fig. 4.4 and Fig. 4.5). Half maximal stimulation of GCS by glucagon occurred at 3.2 ± 0.6 vs. 0.2 ± 0.1 nM in the presence of 10^{-6} M okadaic acid, $p < 0.05$.

Protein kinase A, or cAMP-dependent protein kinase, is a major kinase responsible for phosphorylating many cytosolic proteins. In the liver, glucagon is known to activate protein-kinase A. Therefore, the question arises to whether cAMP-mediated phosphorylation of cytosolic protein is responsible for the activation of GCS, which is a mitochondrial enzyme. Sp-cAMPS, a cell-permeable agonist for protein kinase A, and Rp-8-Br-cAMPS, a cell-permeable antagonist for protein kinase A, were used to examine the regulation of GCS. Both of these

Fig. 4.2 Effects of submaximal okadaic acid on glucagon stimulation of GCS flux in isolated hepatocytes

Okadaic acid (0.01 μ M) was preincubated with the isolated hepatocytes for 20 minutes at 37°C. This was followed by a 30 minute incubation period with glucagon and 1-¹⁴C glycine. A dose response curve for the glucagon stimulation of GCS flux without okadaic acid was done as a control. The basal rate of GCS flux is 1.3 ± 0.3 nmoles/ 30 min/ mg dry wt. Results are expressed as the percentage of the unstimulated rate, without added okadaic acid. Results are means \pm SD, n = 3.

Fig. 4.2

EFFECT OF 10 nM OKADAIC ACID ON THE GLUCAGON STIMULATION
OF FLUX THROUGH GCS IN ISOLATED HEPATOCYTES

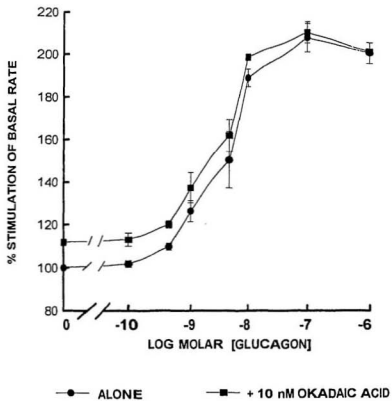


Fig. 4.3 Effect of submaximal okadaic acid on the glucagon stimulation of glycogenolysis

A dose-response curve for glucagon was carried out with (■) and without (●) preincubating the hepatocytes for 20 minutes with 0.01 μ M okadaic acid at 37°C. Glycogenolysis was measured as nmoles of glucose produced/ 30 min/ mg dry wt. Results are expressed as percentage of the unstimulated basal rate without added okadaic acid and are means of three separate experiments \pm SD. The basal rate of glucose production is 202 ± 10 nmoles glucose/ 30 min/ mg dry wt.

Fig. 4.3

EFFECT OF 10 nM OKADAIC ACID ON THE GLUCAGON STIMULATION
OF GLYCOGENOLYSIS IN ISOLATED HEPATOCYTES

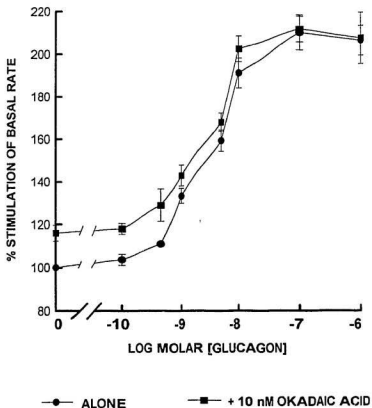


Fig. 4.4 Effect of maximal concentration of okadaic acid on glucagon-stimulated GCS flux in isolated hepatocytes

Isolated hepatocytes were preincubated at 37°C for 20 minutes with (■) and without (●) 10^{-6} M okadaic acid before adding 0.3 mM 1- 14 C glycine and different concentrations of glucagon. The 14 CO₂ released is expressed as a percentage of the basal rate without added okadaic acid. The basal rate for GCS flux is 1.7 ± 0.4 nmoles/ 30 min/ mg dry wt. Results are the means of three separate experiments \pm SD.

Fig. 4.4

EFFECT OF MAXIMAL DOSE OF OKADAIC ACID ON THE
GLUCAGON-STIMULATED FLUX THROUGH GCS

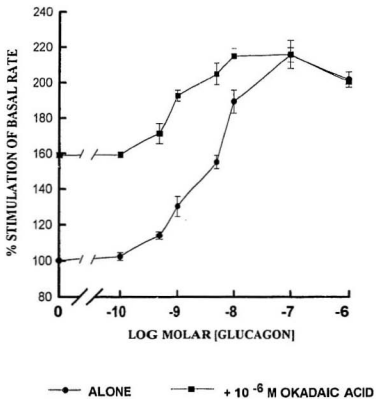
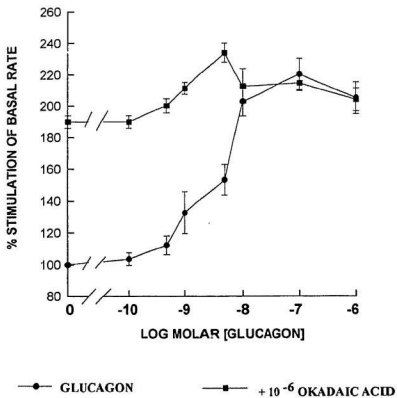


Fig. 4.5 Effect of maximal okadaic acid on the glucagon stimulation of glycogenolysis

Glucose production was determined both in the presence (■) and absence (●) of 10^{-6} M okadaic acid. The basal rate of glucose production is 210 ± 21 nmoles glucose/ 30 min/ mg dry wt. Results are presented as the percentage of the unstimulated rate and are means \pm SD, n=3.

Fig. 4.5

**EFFECT OF MAXIMAL DOSE OF OKADAIC ACID ON THE GLUCAGON
STIMULATION OF GLYCOGENOLYSIS IN ISOLATED HEPATOCYTES**



compounds have already been used to examine the regulation of glycogenolysis and gluconeogenesis in isolated hepatocytes (Rothermel *et al.*, 1983; 1984a; Meserve *et al.*, 1986; Marks and Parker-Botelho, 1986). Sp-cAMPS stimulated the flux through GCS and glycogenolysis whereas Rp-8-Br-cAMPS was without effect on either parameter (Fig. 4.6) A control experiment showed that neither Rp-8-Br-cAMPS nor Sp-cAMPS has an effect on the GCS flux in isolated mitochondria (Table 4.2). Thus the effect of Sp-cAMPS in isolated hepatocytes is attributed to its activation of PK-A.

The question that was next considered is whether Rp-8-Br-cAMPS would be able to inhibit the Sp-cAMPS stimulated GCS flux in isolated hepatocytes. Rp-8-Br-cAMPS did inhibit Sp-cAMPS stimulation of both flux through GCS and glycogenolysis. A maximum inhibition was found at 10^{-5} M Rp-8-Br-cAMPS (Fig. 4.7). Competition between these two CAMP analogues was also examined in experiments in which the concentration of the antagonist was varied. Preincubation of isolated hepatocytes with Rp-8-Br-cAMPS (10^{-5} M) before they were challenged with different concentrations of Sp-cAMPS caused a significant inhibition in the Sp-cAMPS-stimulated GCS flux (Fig. 4.8). A similar inhibition of Sp-cAMPS-stimulated glycogenolysis was observed at the same concentration of Rp-8-Br-cAMPS (Fig. 4.9). These results show that Rp-8-Br-cAMPS does antagonize

Fig. 4.6 Effect of cAMP analogues on the flux through GCS and glycogenolysis in isolated hepatocytes

Isolated hepatocytes were preincubated with varying concentrations of Rp-8-Br-cAMPS for 20 minutes and then were incubated with 1-¹⁴C glycine for a further 30 minutes. In experiments with the stimulatory Sp-cAMPS, the hepatocytes were preincubated alone for 20 minutes and then 1-¹⁴C glycine and Sp-cAMPS were added and incubation continued for a further 30 minutes. The basal rate of GCS flux was 1.7 ± 0.3 nmoles/ 30 min/ mg dry wt. and half maximal stimulation of the flux through GCS occurred at 215 ± 69.5 nM Sp-cAMPS. The basal rate of glucose production was 170 ± 34 nmoles/ 30 min/ mg dry wt. Half maximal stimulation of glycogenolysis occurred at 181 ± 22 nM Sp-cAMPS. Results are presented as the percentage of the unstimulated rate and are means \pm SD of three separate experiments.

Fig. 4.6

EFFECT OF Sp-cAMPS AND Rp-8-Br-cAMPS ON GLYCOGENOLYSIS
AND FLUX THROUGH GCS

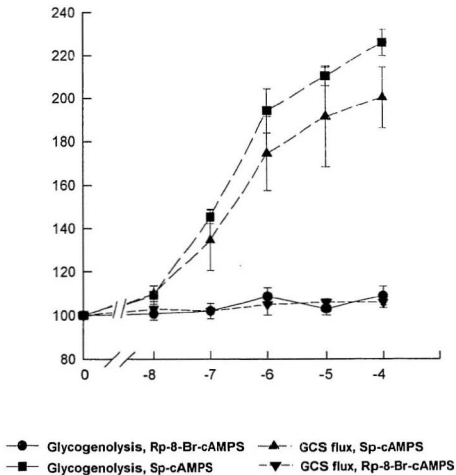


Table 4.2 Effects of Sp-cAMPS and Rp-8-Br-cAMPS on the flux through GCS in isolated mitochondria

	Flux through GCS (nmoles ¹⁴ CO ₂ released/ mg mitochondrial protein/ min)
Control, H ₂ O	0.159 ± 0.02
Sp-cAMPS, 10 ⁻⁵ M	0.149 ± 0.01
Rp-8-Br-cAMPS, 10 ⁻⁴ M	0.163 ± .023

Mitochondria were isolated then preincubated alone or with Rp-8-Br-cAMPS for 20 minutes. Then they were incubated for 30 minutes with Sp-cAMPS and 0.3 mM 1-¹⁴C glycine before the reactions were terminated with 30% perchloric acid and ¹⁴CO₂ was trapped and counted. Results are given as nmoles ¹⁴CO₂/ mg mitochondrial protein/ min. and are means ± SD; n = 3.

Fig. 4.7 Effects of Rp-8-Br-cAMPS on the Sp-cAMPS-stimulated GCS flux and glycogenolysis in isolated hepatocytes

Isolated hepatocytes were preincubated with different concentrations of Rp-8-BR-cAMPS for 20 minutes before they were incubated with 10^{-6} M Sp-cAMPS and 0.3 mM $1\text{-}^{14}\text{C}$ glycine for 30 minutes. The basal rate for GCS flux was 2.0 ± 0.2 nmoles/ 30 min/ mg dry wt. and for glucose production, 120 ± 19 nmoles/ 30 min/ mg dry wt. Results were expressed as percentage of the unstimulated rate and are means \pm SD. *p < 0.05 vs. control with no Rp-8-Br-cAMPS, using ANOVA.

Fig. 4.7

EFFECT OF Rp-8-Br-cAMPS ON Sp-cAMPS STIMULATION OF FLUX
THROUGH GCS AND GLYCOGENOLYSIS

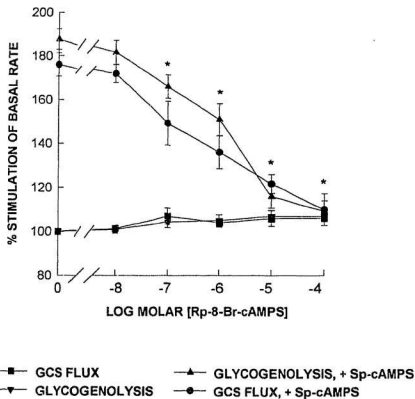


Fig. 4.8 Effect of Rp-8-Br-cAMPS on Sp-cAMPS stimulation of the flux through GCS

A dose-response curve for the Sp-cAMPS stimulation of the flux through GCS was carried out with (■) and without (●) 10^{-5} M Rp-8-Br-cAMPS. The basal rate for GCS flux is 2.2 ± 0.5 nmoles/ 30 min/ mg dry wt. Half maximal stimulation occurred at (■) 198.6 ± 100 and at (●) 902 ± 133 nM Sp-cAMPS. Results are percentage of the unstimulated rate and means of three separate experiments. * $p < 0.05$ vs. control with no Rp-8-Br-cAMPS, by paired t-test.

Fig. 4.8

EFFECT OF Rp-8-Br-cAMPS ON Sp-cAMPS STIMULATION OF
THE FLUX THROUGH GCS

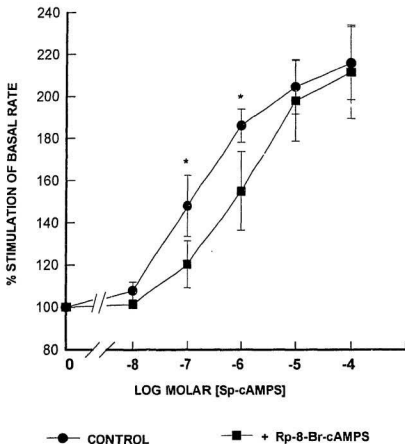
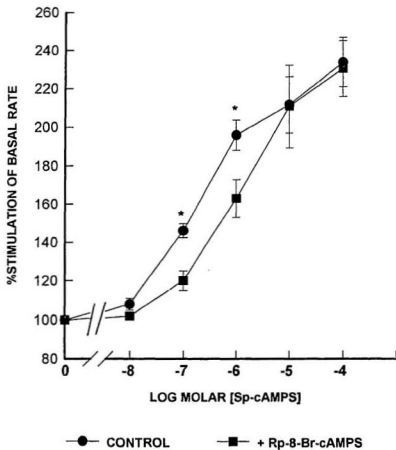


Fig. 4.9 Effect of Rp-8-Br-cAMPS on the Sp-cAMPS stimulation of glycogenolysis in isolated rat hepatocytes

A dose-response curve was carried out for the Sp-cAMPS stimulation of glycogenolysis in the presence and absence of 10^{-5} M Rp-8-Br-cAMPS. The basal rate of glucose production was 159 ± 33 nmoles/ 30 min/ mg dry wt. Half maximal stimulation occurred at 220 ± 33 nM Sp-cAMPS vs. 804 ± 155 nM Sp-cAMPS in the presence of 10^{-5} M Rp-8-Br-cAMPS. The data are expressed as a percentage of the unstimulated rate and are means \pm SD. * $p < 0.05$ vs. Sp-cAMPS with 10^{-5} M Rp-8-Br-cAMPS; by paired t-test.

Fig. 4.9

EFFECT OF Rp-8-Br-cAMPS ON Sp-cAMPS STIMULATION
OF GLYCOGENOLYSIS



The stimulation of GCS by Sp-cAMPS. The effect of Rp-8-Br-cAMPS on the glucagon-stimulated GCS flux and glycogenolysis was determined. Results presented in Fig. 4.10 show no effect of Rp-cAMPS on the glucagon stimulated flux through GCS although it did antagonize glucagon-stimulated glycogenolysis in the same experiment. A dose-response curve for the glucagon stimulation of GCS flux was carried out in the presence and absence of 10^{-5} M Rp-8-Br-cAMPS (Fig. 4.11). No right shift of the glucagon curve was observed. However, when glycogenolysis was measured in these experiments a significant right shift in the glucagon dose response curve was found as shown in Fig. 4.12.

Effect of calcium-mobilizing hormones on the GCS

The effects of vasopressin and angiotensin II on the flux through GCS were examined since these hormones act through increases in intracellular Ca^{2+} concentration and have no effect on cAMP or protein kinase A in the liver (Kirk and Hems, 1974; Hems et al., 1978). Incubation of isolated rat hepatocytes with 10^{-10} - 10^{-6} M vasopressin or angiotensin II resulted in stimulation of the flux through GCS and of glycogenolysis as shown in Fig. 4.13 and Fig. 4.14.

Fig. 4.10 Effect of Rp-8-Br-cAMPS on the glucagon-stimulated flux through GCS and glycogenolysis in isolated hepatocytes

Isolated hepatocytes were preincubated with different concentrations of Rp-8-Br-cAMPS before they were challenged with 10 nM glucagon. The basal rate of GCS flux is 2.0 ± 0.2 nmoles/ 30 min/ mg dry wt. and basal rate of glucose production, 156 ± 39 nmoles/ 30 min/ mg dry wt. Results are presented as percentage of the unstimulated rate and are means of three experiments \pm SD. * $p < 0.05$ vs. controls with no Rp-8-Br-cAMPS; paired t-test.

Fig. 4.10

EFFECT OF Rp-8-Br-cAMPS ON THE GLUCAGON STIMULATION
OF GLYCOGENOLYSIS AND GCS FLUX

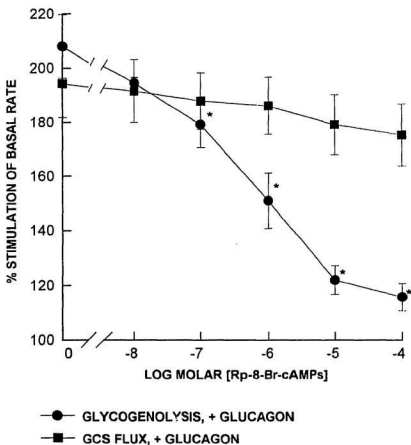


Fig. 4.11 Effect of Rp-8-Br-cAMPS on glucagon dose-response curve on the flux through GCS in isolated hepatocytes

A glucagon dose-response curve was done with (■) and without (●) preincubation of the isolated hepatocytes with Rp-8-Br-cAMPS. The basal rate of GCS flux is 2.3 ± 0.9 nmoles/ 30 min/ mg dry wt. Half maximal stimulation occurred at 2.0 ± 0.3 nM vs. 2.7 ± 0.3 nM glucagon, respectively, in the absence and presence of Rp-8-Br-cAMPS. Results are means \pm SD, n = 3.

Fig. 4.11

EFFECT OF Rp-8-Br-cAMPS ON GLUCAGON STIMULATION
OF FLUX THROUGH GCS

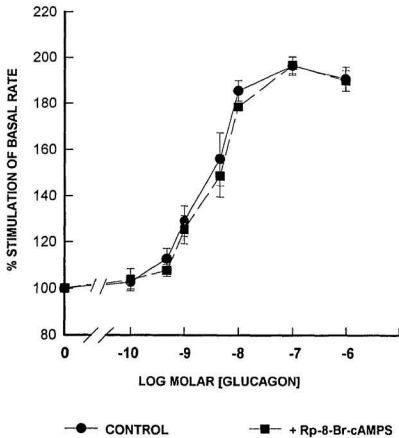


Fig. 4.12 Effect of Rp-8-Br-cAMPS on glucagon-stimulated glycogenolysis in isolated hepatocytes

A dose-response curve for glucagon was carried out with (■) and without (●) 10^{-5} M Rp-8-Br-cAMPS. The basal rate of glucose production is 205 ± 14 nmoles/ 30 min/ mg dry wt. Results are means \pm SD, n = 3. Half maximal stimulation is (■) 2.26 ± 0.17 nM vs. (●) 5.04 ± 0.96 nM, $p < 0.05$, paired t-test.

Fig. 4.12

EFFECT OF Rp-8-Br-cAMPS ON GLUCAGON STIMULATION
OF GLYCOGENOLYSIS IN ISOLATED RAT HEPATOCYTES

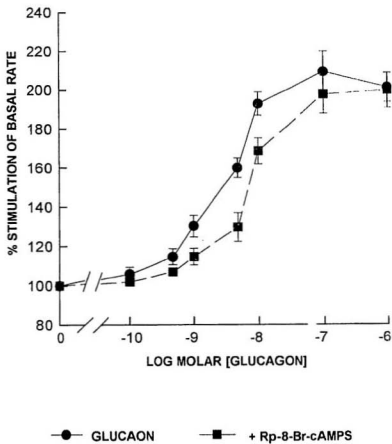


Fig. 4.13 Effect of vasopressin on the flux through GCS and glycogenolysis in isolated hepatocytes

The basal rate of GCS flux is 2.2 ± 0.8 nmoles/ 30 min/ mg dry wt. The basal rate of glucose production is 139 ± 53 nmoles/ 30 min/ mg dry wt. Results are presented as percentage of the unstimulated rate of both GCS flux and glycogenolysis. Results are means of three experiments \pm SD.

Fig. 4.13

EFFECT OF VASOPRESSIN ON THE FLUX THROUGH GCS
AND GLYCOGENOLYSIS IN ISOLATED RAT HEPATOCYTES

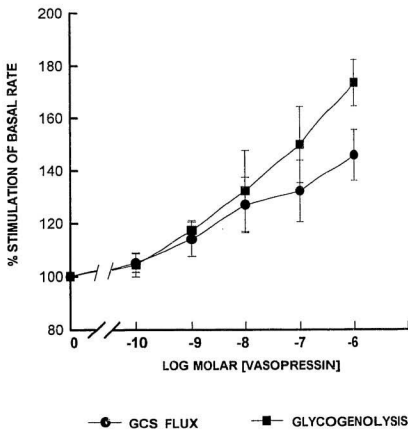
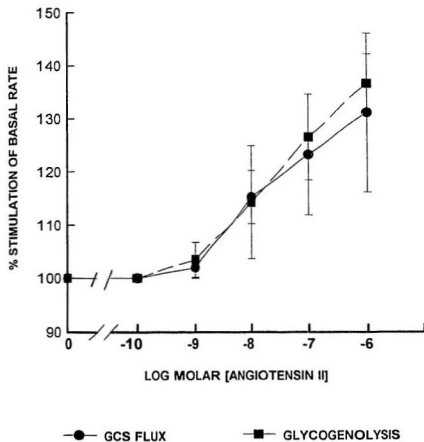


Fig. 4.14 Effect of angiotensin II on the flux through GCS and glycogenolysis in isolated hepatocytes

Results are expressed as percentage of the unstimulated rate. The basal rate for GCS flux is 1.8 ± 0.2 nmoles/ 30 min/ mg dry wt. The basal rate for glucose production is 101 ± 16 nmoles/ 30 min/ mg dry wt. Results are means \pm SD of three separate experiments.

Fig. 4.14

STIMULATION OF THE FLUX THROUGH GCS AND GLYCOGENOLYSIS
BY ANGIOTENSIN II IN ISOLATED RAT HEPATOCYTES



Role of protein kinase C in the regulation of GCS

The question of a possible role of protein kinase C in the glucagon-stimulated flux through GCS was examined. A dose-response curve for the flux through GCS and glycogenolysis was done with phorbol 12-myristate 13-acetate (PMA) and phorbol 12-myristate 13-acetate 4-O-methyl ether (PMA 4-O-methyl ether) in isolated hepatocytes as shown in Fig. 4.15. PMA, a tumour promoting phorbol ester, is a structural analogue of diacylglycerol that activates protein kinase C (Houslay, 1991). PMA 4-O-methyl ether interacts poorly with protein kinase C (Tronchere et al., 1993); therefore it was used in these experiments as a control. No stimulation was found with either PMA or PMA 4-O-methyl ether.

We then examined if PMA would have an effect on the glucagon-stimulated GCS flux and glycogenolysis. Isolated hepatocytes were preincubated with PMA or PMA 4-O-methyl ether before they were challenged with glucagon. PMA had no effect on stimulation of the flux through GCS (Fig. 4.16) or glycogenolysis (Fig. 4.17) by glucagon as indicated by the magnitude of the maximal stimulation and half maximal stimulation.

Fig. 4.15 Effect of PMA on the flux through GCS and glycogenolysis in isolated hepatocytes

Isolated hepatocytes were preincubated for 20 minutes with PMA before the reaction was initiated by addition of 0.3 mM 1-¹⁴C glycine. Control incubations were carried out with PMA methyl ether. The basal rate of GCS flux is 1.5 ± 0.2 nmoles/ 30 min/ mg dry wt. The basal rate of glucose production is 195 ± 35 nmoles/ 30 min/ mg dry wt. Results are means of three separate experiments \pm SD.

Fig. 4.15

EFFECT OF PMA ON THE FLUX THROUGH GCS AND
GLYCOGENOLYSIS IN ISOLATED HEPATOCYTES

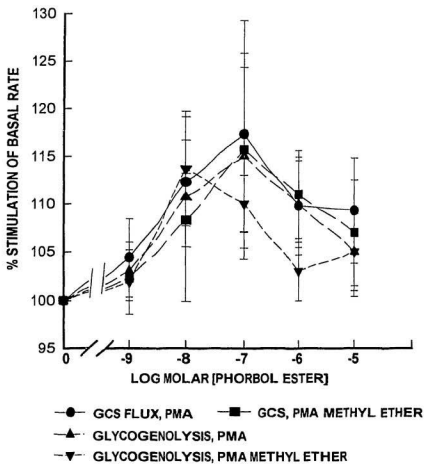


Fig. 4.16 Effect of PMA on the glucagon stimulation of GCS flux in isolated hepatocytes

Isolated hepatocytes were preincubated in the presence or absence of PMA and PMA methyl ether, after which they were incubated with varying concentrations of glucagon and 0.3 mM 1-¹⁴C glycine. The basal rate is 1.3 ± 0.6 nmoles/ 30 min/ mg dry wt. Results are presented as percentage of the unstimulated rate and are means \pm SD, n = 3. Half maximal stimulations are (●) 2.2 ± 0.4 (▲) 2.6 ± 0.2 nM (■) 2.1 ± 0.26 nM, p > 0.05, paired t-test.

Fig. 4.16

EFFECT OF PMA ON GLUCAGON STIMULATION OF FLUX THROUGH GCS

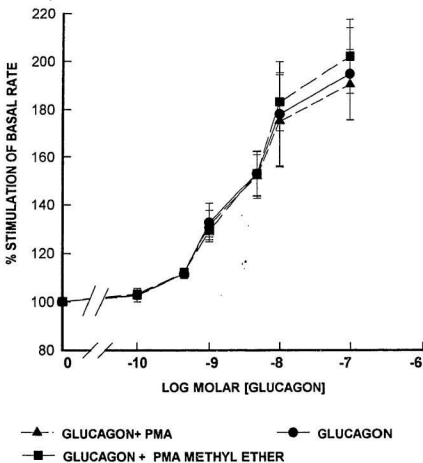
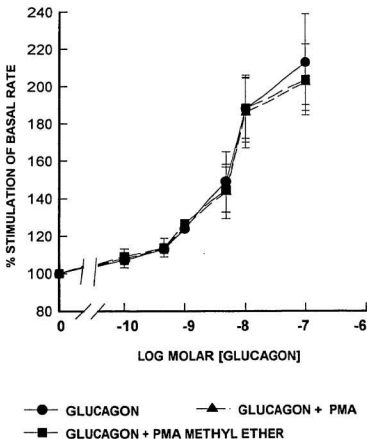


Fig. 4.17 Effect of PMA on glucagon-stimulated glycogenolysis in isolated rat hepatocytes

The isolated hepatocytes were preincubated in the presence or absence of PMA or PMA methyl ether. Results are percentage of the unstimulated rate and are means \pm SD of three separate experiments. The basal rate of glucose production is 173 ± 50 nmoles/ 30 min/ mg dry wt. Half maximal concentrations are (●) 3.5 ± 0.4 nM (■) 3.6 ± 0.6 nM (▲) 3.3 ± 0.2 nM, $p > 0.05$, paired t-test.

Fig. 4.17

EFFECT OF PMA ON GLUCAGON STIMULATION OF
GLYCOGENOLYSIS



Discussion

Although the regulation of the GCS enzyme system by hormones has been established, the mechanism by which the signal is transmitted from the glucagon receptor through the cytoplasm into mitochondria is not known.

Phosphorylation/ dephosphorylation events play important roles in metabolic regulation, particularly in glucagon action. Therefore, the possibility of the involvement of phosphorylated proteins in the regulation of GCS was examined by using the cytosolic protein phosphatase inhibitor okadaic acid. Okadaic acid's specific effect is to inhibit PP1 and PP2A in the cytosol leading to increase in the level of phosphorylated proteins (Cohen and Cohen, 1989). Results shown in Fig. 4.1 and Fig. 4.2 suggest that increase in cytosolic protein phosphorylation, produced by okadaic acid, causes a significant increase in the flux through GCS and glycogenolysis. Results from table 4.1 confirm that okadaic acid had no direct effect on GCS flux in isolated mitochondria. In isolated hepatocytes, Haystead *et al.*, (1989) reported a 3 fold increase in the level of cytoplasmic phosphorylated proteins in response to okadaic acid with no increase in the microsomal fraction. However, in their experiments, the mitochondrial fraction was not examined to detect any increase in the level of phosphorylated protein.

The finding that okadaic acid mimics hormonal actions on the flux through GCS raised the question of possible involvement of phosphorylation/dephosphorylation of cytoplasmic proteins in the hormonal signalling to the mitochondrial enzyme GCS. The data in Fig. 4.2 show that a submaximal concentration of okadaic acid did not produce a left shift in the glucagon curve. Okadaic acid at a concentration of 10^{-6} M was able to produce a significant left shift of the glucagon dose-response curve (Fig. 4.4). This result suggests that cytosolic protein phosphorylation may be an important element in the glucagon stimulation of the flux through GCS.

Protein kinase A, or cAMP-dependent protein kinase, is responsible for the phosphorylation of many cytosolic proteins. In addition, Protein kinase A is implicated in glucagon action. Therefore, it is possible that cAMP-induced phosphorylation may also be involved in the regulation of GCS. Sp-cAMPS and Rp-8-Br-cAMPS, the cAMP analogues, were used to examine this hypothesis. Sp-cAMPS significantly stimulated the GCS flux while Rp-8-Br-cAMPS had no effect on the GCS flux or on the basal rate of $^{14}\text{CO}_2$ production. Furthermore, the Sp-cAMPS-stimulated flux through GCS is inhibited by the protein kinase antagonist, Rp-8-Br-cAMPS which shows that stimulation of the cytosolic cAMP-dependent protein kinase can stimulate the flux through GCS.

The possibility of involvement of cAMP-dependent

phosphorylation in the glucagon effect on GCS was examined. A right shift in the glucagon dose-response curve caused by Rp-8-Br-cAMPS would strongly support the idea that glucagon is stimulating the flux through GCS via cAMP-dependent increases in phosphorylation of cytoplasmic proteins. Our results show that there is no significant change in the glucagon response curve. However, Rp-8-Br-cAMPS did antagonize the glucagon-stimulated glycogenolysis which is consistent with results of Rothermel *et al.*, (1984a).

A possible explanation for the absence of an inhibitory effect of Rp-8-Br-cAMPS on glucagon-stimulated flux through GCS would be that glucagon stimulation leads to increase in both cAMP and Ca^{2+} (Wakelam *et al.*, 1986; Jelinek *et al.*, 1993), in the liver. Rp-8-Br-cAMPS may inhibit the increase in cAMP concentration but would not affect the increase in calcium concentration which may be an important element in the glucagon regulation of GCS. The observation that phosphorylation of some glucagon targets can be stimulated by agents that increase intracellular Ca^{2+} concentration (Garrison and Wagner, 1982; Garrison *et al.*, 1984) raised the possibility that Ca^{2+} /calmodulin-sensitive protein kinases may also play a role in the response of hepatocytes to glucagon.

The role of intracellular Ca^{2+} in the regulation of GCS was examined with the calcium mobilizing hormones, vasopressin and

angiotensin II. They both stimulated the flux through GCS and glycogenolysis. This is consistent with the results presented by Hems *et al.*, (1978) and Jois *et al.*, (1990a). Hems *et al.*, (1978) reported rapid stimulation of glycogenolysis by (10 pM-10 nM) vasopressin and (1 nM-0.1 μ M) angiotensin II in hepatocyte suspensions. Jois *et al.*, (1990a) reported stimulation of the flux through GCS in isolated rat liver perfused with 100 nM vasopressin.

The question as to whether protein kinase C is involved in the regulation of the GCS enzyme system was examined using phorbol esters. Results in Fig. 4.19 show no effect of PMA or PMA 4-O-methyl ether on the GCS or glycogenolysis, which suggests no role for protein kinase C on the flux through GCS or glycogenolysis. Our results are in agreement with those of Corvera and Garcia-Sainz, (1984) who showed that (10^{-11} - 10^{-6} M) PMA was unable to stimulate glycogenolysis in isolated rat hepatocytes. In addition, there was no effect of PMA on the glucagon stimulation of the flux through GCS or glycogenolysis. This is consistent with Corvera and Garcia-Sainz, (1984) who showed that the stimulation of glycogenolysis by glucagon was unaffected by PMA in isolated rat hepatocytes. Although 0.1-1 μ M PMA has been reported to decrease the accumulation of cAMP induced by glucagon, it did not affect the glucagon-stimulated ureogenesis (Garcia-Sainz

et al., 1985). Dissociation between the cAMP concentrations and the metabolic effects induced by glucagon is evidenced in the presence of phorbol esters. In the perfused rat liver, infusion of PMA for 20 minutes prior to infusion of glucagon did not alter glucagon-stimulated glycogenolysis as measured by the increase in glucose output and decrease in lactate output (Puschel et al., 1993). Therefore, we suggest that protein kinase C does not play a role in the regulation of GCS. However, we should be aware that using phorbol esters, which are structural analogues of diacylglycerol, has some problems. The first problem is that phorbol esters achieve a prolonged activation of protein kinase C and may cause its down-regulation (Nishizuka, 1988). The second problem is that diacylglycerol produced in the cell may activate protein kinase C isoforms selectively, depending upon its fatty acid composition and that such a variety of activity may be different from that achieved by phorbol esters.

Unanswered questions

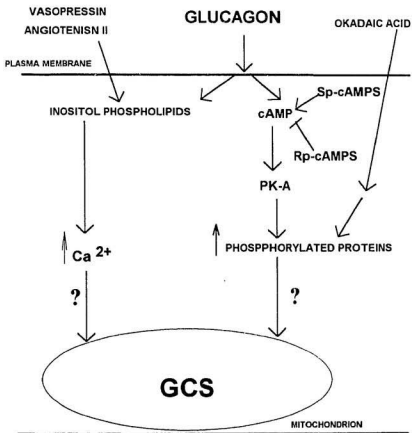
The occupation of the glucagon receptor by glucagon exerts two main effects. The first effect is that it stimulates adenylate cyclase with the consequent rise in [cAMP] (Heyworth and Houslay, 1983) and the increase in the concentration of phosphorylated proteins. The second effect is

an increase in free $[Ca^{2+}]$, as initiated by the stimulation of phosphoinositol turnover (Wakelam *et al.*, 1986; Mine *et al.*, 1988). These events occur via distinct G proteins.

Glucagon was found to stimulate the flux through GCS. However, it is not known exactly which signalling pathway is responsible for the activation of this mitochondrial enzyme, or how the hormonal signal is transmitted to the mitochondria (Fig. 4.18). Therefore we examined the effect of various agents that specifically stimulate or inhibit certain parts of the signalling pathways. Our results demonstrate the involvement of protein phosphorylation, cAMP, and intracellular calcium concentration in the stimulation of the GCS by hormones such as glucagon.

However, a number of unanswered questions still need to be investigated. The identity of phosphorylated proteins and their location within the hepatocytes is not known. The question of how the increase in cytosolic protein phosphorylation can affect the GCS which is located inside the mitochondria remains unanswered and still needs to be examined.

Fig. 4.18 UNANSWERED QUESTIONS



Chapter 5
SUMMARY

Summary

1. Glicentin and oxyntomodulin stimulate the flux through GCS in isolated rat hepatocytes.
2. Glicentin and oxyntomodulin exert their effects in isolated rat hepatocytes through interaction with glucagon receptors.
3. GLP-1 and miniglucagon have no effect on the glycine cleavage system. GLP-1 and miniglucagon are neither agonists nor antagonists for glucagon in isolated hepatocytes.
4. Okadaic acid, the cell-permeable cytosolic protein phosphatase inhibitor, stimulates the flux through GCS.
5. Sp-cAMPS, a protein kinase A agonist, stimulates the flux through GCS in isolated hepatocytes. This stimulation can be inhibited by the protein kinase A antagonist Rp-8-Br-cAMPS. Thus increase in cytosolic protein phosphorylation levels, probably through a cAMP-dependent protein kinase, can cause an increase in the flux through GCS.
6. The glucagon-stimulated flux through GCS is not inhibited by the protein kinase A antagonist, Rp-8-Br-cAMPS although it is able to inhibit the activation of glycogenolysis, a

cytoplasmic process. This suggests that the signalling mechanism involved in transmitting the glucagon signal into mitochondria is more complex and may involve many elements.

7. The calcium-mobilizing hormones, vasopressin and angiotensin II, stimulate the flux through GCS. However, in isolated hepatocytes they did not potentiate the glucagon stimulation of GCS flux at the concentrations tested.

8. Phorbol esters do not increase the flux through GCS on their own, nor do they potentiate the effect of a submaximal dose of glucagon. They do not have an effect on glycogenolysis either.

REFERENCES

REFERENCES

Ahrén, B. and Lundquist, I. (1980) Effects of glicentin on insulin secretion. *Horm. Metab. Res.*, 12: 582-586.

Balaban, J.S. and Blum, J.J. (1982) Hormone-induced changes in NADH fluorescence and O₂ consumption of rat hepatocytes. *Am. J. Phys.*, 242: C172-C177.

Bataille, D., Gespach, C., Coudary, A.M., Rosselin, G. (1981) 'Enteroglucagon': A specific effect on gastric glands isolated from the rat fundus. Evidence for an oxyntomodulin action. *Biosci. Rep.*, 1: 151-155.

Bataille, D., Coudray, A.M., Carlqvist, M., Rosselin, G. and Mutt, V. (1982a) Isolation of glucagon-37 (bioactive enteroglucagon/oxyntomodulin) from porcine jejuno-ileum. Isolation of the peptide. *FEBS Lett.*, 146: 73-78.

Bataille, D., Tatemoto, K., Gespach, C., Jornvall, H., Rosselin, G. and Mutt, V. (1982b) Isolation of glucagon-37 (bioactive enteroglucagon/oxyntomodulin) from porcine jejuno-ileum. Characterization of the peptide. *FEBS Lett.*, 146: 79-86.

Bataille, D., Blache, P., Mercier, F., Jarrousse, C., Kervran, A., Dufour, M., Mangeat, P., Dubrasquet, M., Mallat, A., Lotersztajn, S., Pavoine, C. and Pecker, F. (1988) Glucagon and related peptides: Molecular structure and biological specificity. *Ann. N.Y. Acad. Sci.*, 527: 168-185.

Bell, G.I., Sanchez-Pescador, R., Laybourn, P.J. and Najarian, R.C. (1983a) Exon duplication and divergence in the human preproglucagon gene. *Nature*, 304: 368-371.

Bell, G.I., Santerre, R.F., Mullenbach, G.T. (1983b) Hamster preproglucagon contains the sequence of glucagon and two related peptides. *Nature*, 302: 716-718.

Bender, D.A. (1985) *Amino Acid Metabolism*. (2nd edition) John Wiley and Sons, Inc. Toronto, pp: 95-118.

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

Berridge, M.J. (1984) Inositol trisphosphate and diacylglycerol as second messengers. *Biochem. J.*, 220: 345-360.

Berry, M.N., Edwards, A.M. and Barritt, G.J. (1991a) High yield preparation of isolated hepatocytes from rat liver. In: Laboratory Techniques in Biochemistry and Molecular biology. Vol. 21 (Burdon, R.H. and Van Knippenberg, P.H., eds.) Elsevier, Amsterdam. New York. Oxford.

Bialojan, C. and Takai, A. (1988) Inhibitory effect of a marine-sponge toxin, okadaic acid on protein phosphatases: Specificity and kinetics. *Biochem. J.*, 256: 283-290.

Blache, P., Kervran, A., Dufour, M., Martinez, J., Le-Nguyen, D., Lotersztajn, S., Pavoine, C., Pecker, F. and Bataille, D. (1989) Glucagon is processed to the (19-29) fragment at the level of the hepatocyte membrane. *C. R. Acad. Sci. Paris*, 308: 467-472.

Blache, P., Kervran, A., Dufour, M., Martinez, J., Le-Nguyen, D., Lotersztajn, S., Pavoine, C., Pecker, F. and Bataille, D. (1990) Glucagon-(19-29), a Ca^{2+} pump inhibitory peptide, is processed from glucagon in the rat liver plasma membrane by a thiol endopeptidase. *J. Biol. Chem.*, 265(35): 21514-21519.

Blackmore, P.F., Mojsov, S., Exton, J.H. and Habener, J.F. (1991) Absence of insulinotropic glucagon-like peptide-I(7-37) receptors on isolated rat liver hepatocytes. FEBS Lett., 283(1): 7-10.

Bloom, S.R. and Polak J.M. (1982) The hormonal pattern of intestinal adaptation: A major role for enteroglucagon. Scand. J. Gastroenterol., 17 [Suppl.74]: 93-103.

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

Brosnan, J.T., Jois, M. and Hall, B. (1990) Hormonal regulation of glycine metabolism. In: Amino acids- Chemistry, Biology and Medicine (Lubec, G., Rosenthal, G.A., eds.), pp: 896-902.

Bushfield, M., Murphy, G.J., Lavan, B.E., Parker, P.J., Hurby, V.J., Milligan, G. and Houslay, M.D. (1990) Hormonal regulation of Gi2 α -subunit-phosphorylation in intact hepatocytes. Biochem. J., 268: 449-457.

Carles-Bonnet, C., Jarrousse, C., Niel, H., Martinez, J., Rolland, M. and Bataille, D. (1992) N-acetyl oxyntomodulin₃₀₋₃₇: Pharmacokinetics and activity on gastric acid secretion. Naunyn-Schmiedeberg's Arch. Pharmacol., 345: 57-63.

Carothers, D.J., Pons, G. and Patel, M.S. (1989) Dihydrolipoamide dehydrogenase: Functional similarities and divergent evolution of the pyridine nucleotide-disulphide oxidoreductase. Arch. Biochem. Biophys., 268: 490-425.

Castagna, M. Takai, Y., Kaibuchi, K., Sanom, K., Kikkawa, U. and Nishizuka, Y. (1982) Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumour promoting phorbol esters. J. Biol. Chem., 257: 7847-7851.

Cereba, J.A., Downes, C.P., Hawkins, P.T., Brewster, G., Michell, R.H. and Kirk, C.J. (1983) Rapid breakdown of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate in rat hepatocytes stimulated by vasopressin and other Ca^{2+} -mobilizing hormones. Biochem. J., 212: 733-747.

Code, C.F. (1982) Histamine receptors and gastric acid secretion. In: Pharmacology of Histamine Receptors. (Gannellin, C.R., ed.) West Bristol, UK. Wright, pp: 217-235.

Cohen, P. and Cohen, P.T.W. (1989) Protein phosphatases come of age. *J. Biol. Chem.*, 264: 21435-21438.

Cohen, P., Holmes, C.F.B. and Tsukitani, Y. (1990) Okadaic acid: A new probe for the study of cellular regulation. *TIBS*, 15: 98-102.

Combettes, L., Berthon, B., Binet, A. and Claret, M. (1986) Glucagon and vasopressin interactions on Ca^{2+} movements in isolated hepatocytes. *Biochem. J.*, 237: 675-683.

Corvera, S. and Garcia-Sainz, A. (1984) Phorbol esters inhibit alpha₁ adrenergic stimulation of glycogenolysis in isolated rat hepatocytes. *Biochem. Biophys. Res. Commun.*, 119(3): 1128-1133.

Corvera, S., Huerta-Bahena, J., Pelton, J.T., Hurby, V.J., Trivedi, D. and Garcia-Sainz, J.A. (1984) Metabolic effects and cyclic AMP levels produced by glucagon, (1-N α -Trinitrophenylhistidine, 12-homoarginine) glucagon and forskolin in isolated rat hepatocytes. *Biochem. Biophys. Acta*, 804: 434-441.

Crane, J.K., Campanile, C.P. and Garrison, J.C. (1982) The hepatic angiotensin II receptor. II: Effect of guanine nucleotides and interactions with cyclic AMP production. *J. Biol. Chem.*, 257: 4959-4965.

DeWit, R.J., Hoppe, J., Stec, W.J., Baraniak, K. and Jastroff, B. (1982) Interaction of cAMP derivatives with the stable cAMP-binding site in the cAMP-dependent protein kinase type I. *Eur. J. Biochem.*, 122: 95-99.

Duberasquet, J.M., Bataille, D. and Gespach, C. (1982) Oxyntomodulin (glucagon-37 or bioactive enteroglucagon): A potent inhibitor of pentagastrin-stimulated acid secretion in rat. *Biosci. Rep.*, 2: 391-395.

Elliot, R.M., Morgan, L.M., Tredger, J.A., Deacon, S., Wright, J. and Marks, V. (1993) Glucagon-like peptide-1 (7-36) amide and glucose-dependent insulinotropic polypeptide secretion in response to nutrient ingestion in man: Acute post-prandial and 24-h secretion patterns. *J. Endocrin.*, 138: 159-166.

Ewart, H.S., Jois, M. and Brosnan, J.T. (1992) Rapid stimulation of the hepatic glycine cleavage system in rats fed on a single high-protein meal. *Biochem. J.*, 283: 441-447.

Exton, J.H. (1981) Molecular mechanisms involved in α -adrenergic responses. *Mol. Cell. Endocrinol.*, 23: 233-264.

Exton, J.H. (1988) The roles of calcium and phosphoinositides in the mechanisms of α_1 -adrenergic and other agonists. *Rev. Physiol. Biochem. Pharmacol.*, 111: 117-224.

Fafournoux, P., Rémésy, C. and Demigné, C. (1990) Fluxes and membrane transport of amino acids in rat liver under different protein diets. *Am. J. Physiol.*, 259: E614-E625.

Fehmann, H.C. and Habener, J.F. (1991) Functional receptors for the insulinotropic hormone glucagon-like peptide I (7-37) on a somatostatin secreting cell line. *FEBS Lett.*, 279: 335-340.

Fehmann, H.C., Göke, B., Göke, R., Trautmann, M.E. and Arnold, R. (1989) Synergistic stimulatory effect of glucagon-like peptide-1 (7-36) amide and glucose-dependent insulin-releasing polypeptide on the endocrine rat pancreas. *FEBS Lett.*, 252: 109-112.

Fujiwara, K. and Motokawa, Y. (1983) Mechanism of the glycine cleavage reaction (Steady state kinetic studies of the p-protein-catalyzed reaction). *J. Biol. Chem.*, 258: 8156-8162.

Fujiwara, K., Okamura-Ikeda, K. and Motokawa, Y. (1984) Mechanism of the glycine cleavage reaction: Further characterization of the intermediate attached to H-protein and of the reaction catalyzed by T-protein. J. Biol. Chem., 259: 10664-10668.

Garcia-Sainz, J.A., Mendlovic, F. and Martínez-Olmedo, M.A. (1985) Effects of phorbol esters on α_1 -adrenergic-mediated and glucagon-mediated actions in isolated rat hepatocytes. Biochem. J., 228: 277-280.

Garrison, J.C. and Wagner, J.D. (1982) Glucagon and the Ca^{2+} -linked hormones angiotensin II, norepinephrine and vasopressin stimulate the phosphorylation of distinct substrates in intact hepatocytes. J. Biol. Chem., 257: 13135-13143.

Garrison, J.C., Johnsen, D.E. and Campanile, C.P. (1984) Evidence for the role of phosphorylase kinase, protein kinase C, and other Ca^{2+} sensitive protein kinases in the response of hepatocytes to angiotensin II and vasopressin. J. Biol. Chem., 259: 1180-1185.

Gefel D., Hendrick, G.K., Mojsov, S., Habener, J.F. and Weir, G.C. (1990) Glucagon-like peptide-I analogs: Effects on insulin secretion and adenosine 3',5' monophosphate formation. *Endocrinology*, 126: 2164-2168.

Ghiglione, M., Blazquez, E., Ultenthal, L.O., de Diego, J.G., Alvarez, E., George, S.K. and Bloom, S.R. (1985) Glucagon-like peptide I does not have a role in hepatic carbohydrate metabolism. *Diabetologia*, 28: 920.

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

Gutniak, M., Holst, J.J., Ørskov, C., Ahren, B. and Efendic, S. (1992) Antidiabetogenic effect of glucagon-like peptide-1 (7-36) amide in normal subjects and patients with diabetes mellitus. *N. Engl. J. Med.*, 236: 1316-1322.

Halestrap, A.W. (1986) In: *Hormonal Control of gluconeogenesis*. (Kraus-Friedman, N., ed.) Vol. III, CRC Press, pp: 31-34.

Hampson, R.K., Barron, L.L. and Olson, M.S. (1983) Regulation of the glycine cleavage system in isolated rat liver mitochondria. *J. Biol. Chem.*, 258: 2993-2999.

Hampson, R.K., Taylor, M.K. and Olson, M.S. (1984) Regulation of the glycine cleavage system in the isolated perfused rat liver. *J. Biol. Chem.*, 259: 1180-1185.

Hardie, D.G., Haystead, T.A.J. and Sim, A.T.R. (1991) Use of okadaic acid to inhibit protein phosphatases in intact cells. *Methods in Enzymology*, 201: 469-476.

Hayasaka, K., Kochi, H., Hiraga, K. and Kikuchi, G. (1980) Purification and properties of glycine decarboxylase, a component of the glycine cleavage system, from rat liver mitochondria and immunochemical comparison of this enzyme from various sources. *J. Biochem.*, 88: 1193-1199.

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

Hems, D.A. and Whitton, P.D. (1973) Stimulation by vasopressin of glycogen breakdown and gluconeogenesis in the perfused rat liver. *Biochem. J.*, 136: 705-709.

Hems, D.A., Whitton, P.D. and Ma, G.Y. (1975) Metabolic actions of vasopressin, glucagon and adrenalin in the intact rat. *Biochim. Biophys. Acta*, 411: 155-164.

Hems, D.A., Rodrigues, L.M. and Whitton, P.D. (1976) Glycogen phosphorylase, glucose output and vasoconstriction in the perfused rat liver: Concentration-dependence of actions of adrenaline, vasopressin and angiotensin II. *Biochem. J.*, 160: 367-374.

Hems, D.A., Rodrigues, L.M. and Whitton, P.D. (1978) Rapid stimulation by vasopressin, oxytocin and angiotensin II of glycogen degradation in hepatocyte suspensions. *Biochem. J.*, 172: 311-317.

Heyworth, C.M. and Houslay, M.D. (1983) Challenge of hepatocytes by glucagon triggers a rapid modulation of adenylate cyclase activity in isolated membranes. *Biochem. J.*, 214: 93-98.

Hiraga, K. and Kikuchi, G. (1980a) The mitochondrial glycine cleavage system. *J. Biol. Chem.*, 255: 1161.

Hiraga, K. and Kikuchi, G. (1980b) The mitochondrial glycine cleavage system: Functional association of glycine decarboxylase and aminomethyl carrier protein. *J. Biol. Chem.*, 255: 11671-11676.

Hiraga, K., Kochi, H., Motokawa, G. (1972) Enzyme complex nature of the reversible glycine cleavage system of cock liver mitochondria. *J. Biochem.*, 72: 1285-1289.

Hiraga, K., Kochi, H., Motokawa, Y. and Kikuchi, G. (1977) Enzyme complex nature of the reversible glycine cleavage system of cock liver mitochondria. *J. Biochem.*, 72: 1285-1289.

Hiraga, K., Kochi, H., Howasaka, K., Kikuchi, G. and Nyhan, W.L. (1981) Defective glycine cleavage system in nonketotic hyperglycinemia: Occurance of a less active glycine decarboxylase and an abnormal aminomethyl carrier protein. *J. Clin. Invest.*, 68: 525.

Hjorth, S.A., Adelhorst, K., Pedersen, B.B., Kirk, O. and Schwartz, T.W. (1994) Glucagon and glucagon-like peptide 1: Selective receptor recognition via distinct peptide epitopes. J. Biol. Chem., 269:(48) 30121-30124.

Holst, J.J. (1978) Extrapancreatic glucagons. Digestion, 17: 168-190.

Holst, J.J. (1994) Glucagonlike peptide 1: A newly discovered gastrointestinal hormone. Gastroentology, 107: 1848-1855.

Holst, J.J., Orskov, C., Schwartz, T.W., Buhl, T. and Baldissera, F.G.A. (1986) Proglucagon 78-107, a potent insulinotropic hormone from lower small intestine (abstr.) Diabetologia, 29: 549A.

Holst, J.J., Orskov, C., Vagn Nielsen, O. and Schwartz, T.W. (1987) Truncated glucagon-like peptide I, an insulin-releasing hormone from the distal gut. FEBS Lett., 211(2): 169-174.

Houslay, M.D. (1991) "Crosstalk": A pivotal role for protein kinase C in modulating relationships between signal transduction pathways. Eur. J. Biochem., 195: 9-27.

Hvidberg, A., Nielsen, M., Hilsted, J., Orskov, C. and Holst, J.J. (1994) Effect of glucagon-like peptide-1 (proglucagon 78-107 amide) on hepatic glucose production in healthy man. *Metabolism*, 43: 104-108.

Isaacs, C.E. and Greengard, O. (1980) The effect of hyperphenylalaninemia on glycine metabolism in developing rat brain. *Biochem. J.*, 192: 441-448.

Ishihara, H., Martin, B.L., Brautigan, D.L., Karaki, H., Ozaki, H., Kato, Y., Fusetani, N., Watabe, S., Hashimoto, K., Uemura, D. and Hartshorne, D.L. (1989) Calyculin A and okadaic acid: Inhibitors of protein phosphatase activity. *Biochem. Biophys. Res. Commun.*, 159(3): 871-877.

Ishikawa, E. (1976) The regulation of uptake and output of amino acids by rat tissues. *Adv. Enzy. Regul.*, 14: 117-136.

Jacobsen, H., Demandt, A., Moody, A.J. and Sundby, F. (1977) Sequence analysis of porcine gut GLP-I. *Biochem. Biophys. Acta*, 493: 452.

Jaeken, J., Corbeel, L., Casaer, P., Crochon, H., Eggermont, E. and Eckkels, R. (1977) Dipropylactate (valproate) and glycine metabolism. *Lancet*, 2: 617.

Jarrousse, C., Bataille, D. and Jeanrenaud, B. (1984) A pure enteroglucagon, oxyntomodulin (glucagon 37), stimulates insulin release in perfused rat pancreas. *Endocrinology*, 115(1): 102-105.

Jarrousse, C., Audousset-puech, M.P., Dubrasquet, M., Niel, H., Martinez, J. and Bataille, D. (1985) Oxyntomodulin (glucagon-37) and its terminal octapeptide inhibit gastric acid secretion. *FEBS Lett.*, 188: 81-84.

Jarrousse, C., Niel, H., Audousset-Puech, M.P., Martinez, J. and Bataille D. (1986) Oxyntomodulin and its C-terminal octapeptide inhibit meal-stimulated acid secretion. *Peptides*, 7(S1): 253-256.

Jarrousse, C., Carles-Bonnet, C., Niel, H., Sabatier, R., Audousset-Puech, M.P., Blache, P. Kervarn, A., Martinez, J. and Bataille, D. (1993) Inhibition of gastric acid secretion by oxyntomodulin and its 19-37 fragment in the conscious rat. *Am. J. Phys.*, 264: G816-G823.

Jarvest, R.L., Lowe, G., Baraniak, J. and Stec, W.J. (1982) A stereochemical investigation of the hydrolysis of cyclic AMP and the (Sp)- and (Rp)-diastereomers of adenosine cyclic 3':5'-phosphorothioate by bovine heart and baker's yeast cyclic AMP phosphodiesterases. *Biochem. J.*, 203: 461-470.

Jelinek, L.J., 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. and Kindsvogel, W. (1993) Expression cloning and signaling properties of the rat glucagon receptor. *Science*, 259: 1614-1616.

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

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

Jois, M., Hall, B. and Brosnan, J.T. (1990b) Stimulation of glycine catabolism in isolated perfused liver by calcium mobilizing hormones and in isolated rat liver mitochondria by submicromolar concentrations of calcium. *J. Biol. Chem.*, 265: 1246-1248.

Kanse, S.M., Kreymann, E., Ghatel, M.A. and Bloom, S.R. (1988) Identification and characterization of glucagon-like peptide-1 7-36 amide-binding sites in the rat brain and lung. *FEBS Lett.*, 241: 209-212.

Kawasaki, H., Sato, T. and Kikuchi, G. (1966) A new reaction for glycine biosynthesis. *Biochem. Biophys. Res. Commun.*, 23: 228-233.

Keppens, S. and DeWulf, H. (1975) The activation of liver glycogen phosphorylase by vasopressin. *FEBS Lett.*, 51: 29-32.

Keppens, S. and DeWulf, H. (1976) The activation of liver glycogen phosphorylase by angiotensin II. *FEBS Lett.*, 68: 279-282.

Keppens, S. and DeWulf, H. (1984) Vasopressin and angiotensin control the activity of liver phosphodiesterase. *Biochem. J.*, 222: 277-280.

Keppens, S., Vandenheede, J.R. and DeWulf, H. (1977) On the role of calcium as a second messenger in liver for the hormonally induced activation of glycogen phosphorylase. *Biochem. Biophys. Acta*, 496: 448-457.

Kervran, A., Blache, P. and Bataille, D. (1987) Distribution of oxyntomodulin and glucagon in the gastro-intestinal tract and the plasma of rat. *Endocrinology*, 121: 704-713.

Kervran, A., Dubrasquet, M., Blache, P., Martinez, J. and Bataille, D. (1990) Metabolic clearance rates of oxyntomodulin and glucagon in the rat: Contribution of the kidney. *Regul. Pept.*, 31: 41-52.

Kikkawa, U., Takai, Y., Tanaka, Y., Miyake, R. and Nishizuka, Y. (1983) Protein kinase C as a possible receptor protein of tumour-promoting phorbol esters. *J. Biol. Chem.*, 258: 11442-11445.

Kikuchi, G. (1973) The glycine cleavage system: Composition, reaction mechanism, and physiological significance. *Mol. Cell Biol.*, 1: 169-187.

Kikuchi, G. and Hiraga, K. (1982) The mitochondrial glycine cleavage system: Unique features of the glycine decarboxylation. *Mol. Cell Biochem.*, 45: 137-149.

Kirk, C.J. and Hems, D.A. (1974) Hepatic action of vasopressin: Lack of a role for adenosine-3'.5'-cyclic monophosphate. *FEBS lett.*, 47: 128-131.

Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U. and Nishizuka, Y. (1980) Activation of calcium and phospholipid-dependent protein kinase by DAG, its possible relation to phosphatidylinositol turnover. *J. Biol. Chem.*, 255: 2273-2276.

Klein, L.M. and Sagers, R.D. (1966) Glycine metabolism. I: Properties of the system catalysing the exchange of bicarbonate with the carboxyl-group of glycine in *Peptococcus glycinophilus*. *J. Biol. Chem.*, 241: 197-205.

Kochi, H., Seino, H. and Ono, K. (1986) Inhibition of glycine oxidation by pyruvate, α -ketoglutarate, and branched-chain α -keto acids in rat liver mitochondria: Presence of interaction between the glycine cleavage system and α -keto acid dehydrogenase complexes. *Arch. Biochem. Biophys.*, 249(2): 263-272.

Kojima, H., Suzuki, M., Hidaka, H., Yanaihara, N., Yanaihara, C. and Harano, Y. (1988) Biological activity of oxyntomodulin in hepatocytes. *Biomed. Res.*, 9: 191-194.

Kolvraa, S. (1979) Inhibition of the glycine cleavage system by branched-chain amino acid metabolites. *Pediatr. Res.*, 13: 889-893.

Kreymann, B., Yiangou, Y., Knase, S., Williams, G., Ghatel, M.A. and Bloom, S.R. (1988) Isolation and characterization of GLP-1 (7-36)amide from rat intestine: Elevated levels in diabetic rats. *FEBS Lett.*, 242(1): 167-170.

Larsson, L.I. and Moody, A.J. (1980) Glicentin and inhibitory polypeptide immunoreactivity in endocrine cells of gut and pancreas. *J. Histochem. Cytochem.*, 28(9): 925-933.

Lotersztajn, S., Pavoine, C., Brechler, V., Roche, B., Dufour, M., Le-Nguyen, D., Bataille, D. and Pecker, F. (1990) Glucagon-(19-29) exerts a biphasic action on the liver plasma membrane Ca^{2+} pump which is mediated by G proteins. *J. Biol. Chem.*, 265(17): 9876-9880.

Ma, G.Y. and Hems, D.A. (1975) Inhibition of fatty acid synthesis and stimulation of glycogen breakdown by vasopressin in the perfused mouse liver. *Biochem. J.*, 152: 389-392.

Mallat, A., Pavoine, C., Dufour, M., Lotersztajn, S., Bataille, D. and Pecker, F. (1987) A glucagon fragment is responsible for the inhibition of the liver Ca^{2+} pump by glucagon. *Nature*, 325(12): 620-622.

Marks, J.S. and Parker-Botelho, L.H. (1986) Synergistic inhibition of hepatic glycogenolysis in the presence of insulin and a cAMP antagonist. *J. Biol. Chem.*, 261(6): 2781-2785.

Mauger, J.P., Poggioli, J., Guesdon, F. and Claret, M. (1984) Noradrenaline, vasopressin and angiotensin increase Ca^{2+} influx by opening a common pool of Ca^{2+} channels in isolated rat liver cells. *Biochem. J.*, 221: 121-127.

Mauger, J.P., Poggioli, J. and Claret, M. (1985) Synergistic stimulation of the Ca^{2+} influx in rat hepatocytes by glucagon and the Ca^{2+} -linked hormones vasopressin and angiotensin II. *J. Biol. Chem.*, 260(21): 11635-11642.

Meserve, C.J., Olivieri, M.C. and Botelho, L.H. (1986) Inhibition of hepatic gluconeogenesis by the R_p -diastereomer of adenosine cyclic 3',5'-phosphorothioate. *Biochem. J.*, 237: 463-468.

Mine, T., Kojima, I. and Ogata, E. (1988) Evidence of cyclic AMP-independent action of glucagon on calcium mobilization in rat hepatocytes. *Biochim. Biophys. Acta*, 970: 166-171.

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

Mojsov, S., Heinrich, G., Wilson, I.B., Ravazzola, M., Orci, L. and Habener, J.F. (1986) Preproglucagon gene expression in pancreas and intestine diversifies at the level of post-translational processing. *J. Biol. Chem.*, 261: 11880-11889.

Mojsov, S., Weir, G.C. and Habener, J.F. (1987) Insulinotropin: Glucagon-like peptide-I (7-37) co-encoded in the glucagon gene is a potent stimulator of insulin release in the perfused rat pancreas. *J. Clin. Invest.*, 79: 616-619.

Mojsov, S., Kopezynski, M.G. and Habener, J.F. (1990) Both amidated and nonamidated forms of glucagon-like peptide I are synthesized in the rat intestine and the pancreas. *J. Biol. Chem.*, 265: 8001-8008.

Moody, A.J., Jacobsen, H., Sundby, F., Frandsen, E.K., Paetens, D. and Orci, L. (1977) Heterogeneity of gut glucagon-like immunoreactivity. In: *Glucagon, Its Role in Physiology and Clinical Medicine*. (Foa, P.P., Bajaaj, J.S. and Foa, W.L., eds.) *Excerpta Medica*, Amsterdam, pp: 129-135.

Motokawa, Y. and Kikuchi, G. (1969a) Glycine metabolism by rat liver mitochondria: II. Methylene tetrahydrofolate as the direct one carbon donor in the reaction of glycine synthesis. *J. Biochem.*, 65: 71-83.

Motokawa, Y. and Kikuchi, G. (1969b) Glycine metabolism by rat liver mitochondria: IV. Isolation and characterization of hydrogen carrier protein, an essential factor for glycine metabolism. *Arch. Biochem. Biophys.*, 135: 402-409.

Motokawa, Y. and Kikuchi, G. (1971) Glycine metabolism in rat liver mitochondria. V. Intramitochondrial localization of the reversible glycine cleavage system and serine hydroxymethyl transferase. *Arch. Biochem. Biophys.*, 146: 461-466.

Motokawa, Y. and Kikuchi, G. (1972) Isolation and partial characterization of the components of the reversible glycine cleavage system of rat liver mitochondria. *J. Biochem.*, 72: 1281-1284.

Motokawa, Y. and Kikuchi, G. (1974) Glycine metabolism by rat liver mitochondria: Reconstitution of the reversible glycine cleavage system with partially purified protein components. *Arch. Biochem. Biophys.*, 164: 624-633.

Nakada, H.I. and Sund, L.P. (1958) Glyoxylic acid oxidation by rat liver. *J. Biol. Chem.*, 233: 8-13.

Nathan, D.M., Schreiber, E., Fogel, H., Mojsos, S. and Habener, J.F. (1992) Insulinotropic action of glucagonlike peptide-1 (7-37) in diabetic and nondiabetic subjects. *Diabetes Care*, 15: 270-276.

Nauck, M.A., Kleine, N., Orskov, C., Holst, J.J., Willins, B., Creutzfeldt, W. (1993) Normalization of fasting hyperglycemia by exogenous GLP-I (7-36) amide in type 2 diabetic patients. *Diabetologia*, 36: 741-744.

Neims, A.H. and Hallerman, L. (1962) Specificity of the D-amino acid oxidase in relation to glycine oxidase activity. J. Biol. Chem., 237: PC927-PC978.

Nishizuka, Y. (1984a) The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature, 308: 693-698.

Nishizuka, Y. (1984b) Protein kinases in signal transduction. Trends. Biochem. Sci. Pres. Ed., 9: 163-166.

Nishizuka, Y. (1988) The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature, 334: 661-665.

Novak, V., Wilks, A., Buell, G. And McEwen, S. (1987) Identical mRNA for preproglucagon in pancreas and gut. Eur. J. Biochem., 164: 553-558.

Nyhan, W.L., Borden, M. and Childs, B. (1961) Idiopathic hyperglycinemia: A new disorder of amino acid metabolism. II. The concentrations of other amino acids in the plasma and their modification by the administration of leucine. Pediat., 27: 539-550.

O'Brien, W.E. (1978) Inhibition of glycine synthase by branched-chain α -keto acids: A possible mechanism for abnormal glycine metabolism in ketotic hyperglycinemia. Arch. Biochem. Biophys., 189: 291-297.

Ohneda, A. (1987) Response of plasma glicentin to intraduodenal administration of glucose in piglets. Diabetes Res. Clin. Pract., 3: 97-102.

Ohneda, A., Ohneda, K., Nagasaki, T. and Sasaki, K. (1995) Insulinotropic action of human glicentin in dogs. Metabolism, 44(1): 47-51.

Orskov, C. (1992) Glucagon-like peptide-1, a new hormone of the entero-insular axis. Diabetologia, 35: 701-711.

Orskov, C. and Poulsen, S.S. (1991) Glucagonlike peptide-1 (7-36) amide receptors only in islets of Langerhans, autoradiographic survey of extracerebral tissues in rats. Diabetes, 40: 1292-1296.

Orskov, C., Holst, J.J., Knuhtsen, S., Baldissera, F.G.A., Poulsen, S.S. and Nielsen, O.V. (1986) Glucagon-like peptides, GLP-1 and GLP-2, predicted products of the glucagon gene, are secreted separately from pig small intestine but not pancreas. *Endocrinology*, 119: 1467.

Orskov, C., Holst, J.J. and Nielsen, O.V. (1988) Effect of truncated glucagon-like peptide-1 (proglucagon 78-107 amide) on somatostatin secretion from pig pancreas, antrum and stomach. *Endocrinology*, 123: 2009-2013.

Orskov, C., Beeersani, M., Johnsen, A.H., Hojrup, P. and Holst, J.J. (1989) Complete sequences of glucagon-like peptide-1 from human and pig small intestine. *J. Biol. Chem.*, 264: 12326-12829.

Orskov, C., Andreassen, J. And Holst, J.J. (1992) All products of proglucagon are elevated in plasma from uremic patients. *J. Clin. Endocrinol. Metab.*, 74: 379-384.

Orskov, C., Wettergren, A. and Holst, J.J. (1993) Biological effects and metabolic rates of glucagonlike peptide-1 (7-36) amide and glucagon-like peptide-1 (7-37) in healthy subjects are indistinguishable. *Diabetes*, 42: 658-661.

Orskov, C., Rabenhøj, L., Kofod, H., Wetergren, A. and Holst, J.J. (1994) Production and secretion of amidated and glycine-extended glucagon-like peptide-1 (GLP-I) in man. *Diabetes*, 43: 535-539.

Parker-Botelho, L.H., Rothermel, J.D., Coombs, R.V. and Jastorff, B. (1988) cAMP analog antagonists of cAMP action. *Methods in Enzymology*, 159: 159-172.

Pertoft, H., Rubin, K., Kjellen, L., Laurent, T.C. and Klingeborn, B. (1977) The viability of cells grown or centrifuged in a new density gradient medium, Percoll(TM). *Exp. Cell Res.*, 110: 449-457.

Pertoft, H., Hirtenstein, M. and Kågedal, L. (1979) Cell separation in a new density gradient medium, Percoll. In: *Cell populations; Methodological Surveys (B) Biochemistry* (Reid, E. ed.), vol. 9. Chichester: Ellis Horwood Ltd, pp: 67.

Pittner, R.A. and Fain, J.N. (1990) Vasopressin and norepinephrine stimulation of inositol phosphate accumulation in rat hepatocytes are modified differently by protein kinase C and protein kinase A. *Biochim. Biophys. Acta*, 1043: 211-217.

Post, S.R., Rubinstein, P.G. and Tager, H.S. (1993) Mechanism of action of des-his¹-[Glu]glucagon amide, a peptide antagonist of the glucagon receptor system. Proc. Natl. Acad. Sci. USA, 90: 1662-1666.

Puschel, G.P., Miura, H., Rube, F.N. and Jungermann, K. (1993) Inhibition by the protein kinase C activator, 4- β -phorbol 12-myristate 13-acetate of the prostaglandin F_{2 α} -mediated and noradrenaline-mediated but not glucagon-mediated activation of glycogenolysis in rat liver. Eur. J. Biochem., 217: 305-311.

Ravazzola, M., Siperstein, A., Moody, A.J., Sundby, F., Jacobsen, H. and Orci, L. (1979a) Glicentin immunoreactive cells, their relationship to glucagon-producing cells. Endocrinology, 105: 499-508.

Ravazzola, M., Siperstein, A., Moody, A.J., Sundby, F., Jacobsen, H. and Orci, L. (1979b) Glicentin-precursor of glucagon. Life Sci., 25: 287-290.

Reinhart, P.H., Taylor, W.M. and Bygrave, F.L. (1984) The action of α -adrenergic agonists on plasma-membrane calcium fluxes in perfused rat liver. Biochem. J., 220: 43-50.

Richert, D.A., Amberg, R. and Wilson, M. (1962) Metabolism of glycine by avian liver. J. Biol. Chem., 237: 99-103.

Robinson, J.L., Foustock, S., Chanez, M., Bois-Joyeux, B. and Peret, J. (1981) Circadian variation of liver metabolites and amino acids in rats adapted to a high protein, carbohydrate-free diet. J. Nutr., 111: 1711-1720.

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

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

Rothermel, J.D., Perillo, N.L., Marks, J.S., and Botelho, L.H. (1984b) Effects of the specific cAMP antagonist, (R_p)-Adenosine Cyclic 3',5'-Phosphorothioate, on the cAMP-dependent protein kinase-induced activity of hepatic glycogen phosphorylase and glycogen synthase. J. Biol. Chem., 259: 15294-15300.

Ruiz-Grande, C., Alarcon, C., Alcantra, A., Castilla, C., Lopez-Novoa, J.M., Villaneuva-Penacillo, M. and Valverde, I. (1993) Renal catabolism of truncated glucagon-like peptide I. *Horm. Metab. Res.*, 25: 612-616.

Sagers, R.D. and Gunsalus, I.C. (1961) Intermediary metabolism of *Diplococcus glycinophilus*: I. Glycine cleavage and one-carbon interconversions. *J. Bacteriol.*, 81: 541-549.

Sato, T., Kochi, H., Motokawa, Y., Kawasaki, H. and Kikuchi, G. (1969) Glycine metabolism by rat liver mitochondria: II. Synthesis of two molecules of glycine from one molecule each of serine, bicarbonate, and ammonia. *J. Biochem.*, 65: 63-70.

Schauder, P. (1984) Effect of starvation, dietary protein, and pancreatic hormones on branched chain keto acid blood levels in man. In: *Branched Chain Amino and keto Acids in Health and Disease*. (Adibi, S.A., Fekl, W., Langenbeck, U. and Schauder, P., eds.), Karger, Basel, pp: 228-241.

Schjoldager, B.T.G., Balissera, F.G.A., Mortensen, P.E., Holst, J.J. and Christiansen, J. (1988) Oxyntomodulin: A potential hormone from the distal gut. Pharmacokinetics and effects on gastric acid and insulin secretion in man. Eur. J. Clin. Invest., 18: 499-503.

Schjoldager, B., Mortensen, P.E., Myhre, J., Christiansen, J. and Holst, J.J. (1989) Oxyntomodulin from distal gut: Role in regulation of gastric and pancreatic functions. Dig. Dis. Sci., 34: 1411-1419.

Schwoch, G. and Helz, H. (1977) Protein-bound adenosine 3':5'-monophosphate in liver of glucagon-treated rats: Determination of half maximal binding in vivo correlation with protein kinase activity. Eur. J. Biochem., 76: 269-276.

Siegel, E.G., Schulze, A., Schmidt, W.E., Creutzfeldt, W. (1992) Comparison of the effect of GIP or GLP-I (7-36) amide on insulin release from rat pancreatic islets. Eur. J. Clin. Invest., 22: 154-157.

Smedsrod, B. and Pertoft, H. (1985) Preparation of pure hepatocytes and reticuloendothelial cells in high yield from a single rat liver by means of Percoll centrifugation and selective adherence. J. Leuk. Biol., 38: 213-230.

Snell, K. (1984) Enzymes of serine metabolism in normal developing and neoplastic rat tissues. *Enzyme Regul.*, 22: 325-408.

Stegink, L.D., Bell, E.F., Dabees, T.T., Andersen, D.W., Zike, W.L. and Filer, L.J. (1983) In amino acids: Metabolism and medical applications. (Blackburn, G.L., Grant, J.P. and Young, V.R., eds.), John Wright, PSG Inc., pp: 123-146.

Stubbs, M., Kirk, C.J. and Hems, D.A. (1976) Role of extracellular calcium in the action of vasopressin on hepatic glycogenolysis. *FEBS lett.*, 69: 199-202.

Sugano, T., Shiota, M., Tanaka, T., Miyamae, Y., Shimada, M. and Oshino, N. (1980) Intracellular redox state and stimulation of gluconeogenesis by glucagon and norepinephrine in perfused rat liver. *J. Biochem.*, 87: 153-166.

Suganuma, M., Fujiki, H., Suguri, H., Yoshizaura, S., Hirota, M., Nakaysau, M., Ojika, M., Wakamatsu, K., Yamado, K. and Sugimura, T. (1988) Okadaic acid: An additional non-phorbol 12-tetradecanoate 13-acetate type tumour promotor. *Proc. Natl. Acad. Sci. USA.*, 85: 1768.

Sundby, F., Jacobsen, H. and Moody, A.J. (1975) Purification and characterization of a protein from porcine gut with glucagon-like immunoreactivity. *Horm. Metab. Res.*, 8: 366.

Suzuki, S., Kawai, K., Ohashi, S., Wantabe, Y. and Yamashita, K. (1992) Interaction of glucagon-like peptide I (7-36) amide and gastric inhibitory polypeptide or cholecystokinin on insulin and glucagon secretion from the isolated perfused rat pancreas. *Metabolism*, 4: 359-363.

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

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

Thomas, A.P., Marks, J.S., Coll, K.E. and Williamson, J.R. (1983) Quantitation and early kinetics of inositol lipid changes induced by vasopressin in isolated and cultured hepatocytes. *J. Biol. Chem.*, 258: 5716-5725.

Thomas, A.P., Alexander, J. and Williamson, J.R. (1984) Relationship between inositol polyphosphate production and the increase of cytosolic free Ca^{2+} induced by vasopressin in isolated hepatocytes. *J. Biol. Chem.*, 259: 5574-5584.

Tronchere, H., Terce, F., Record, M. and Chap, H. (1993) Phorbol myristate acetate stimulates [^3H]choline incorporation into phosphatidylcholine independently of the 'de novo' pathway in Krebs-II ascitic cells: A unique effect of phorbol ester on choline uptake. *Biochem. J.*, 293(3): 739-744.

Thornes, B. (1992) Expression cloning of the pancreatic B cell receptor for the gluco-incretin hormone glucagon-like peptide I. *Proc. Natl. Acad. Sci. USA*, 89: 8641-8645.

Thornes, B. and Waeber, G. (1993) Glucagon-like peptide I and the control of insulin secretion in the normal state and in NIDDM. *Diabetes*, 42: 1219-1225.

Unger, R.H. and Orci, L. (1990) Glucagon. In: *Diabetes Mellitus*. (Porte, D.Jr. and Riffkin, H., eds.) N.Y., Elsevier, 1990, pp: 104-120.

Unson, C.G. and Merrifield, R.B. (1994) Identification of an essential serine residue in glucagon: Implication for an active site triad. Proc. Natl. Acad. Sci. USA, 91: 454-458.

Unson, C.G., Andru, D., Gruzenda, E.M. and Merrifield, R.B. (1987) Synthetic peptide antagonists of glucagon. Proc. Natl. Acad. Sci. USA, 84: 4083-4087.

Unson, C.G., Gruzenda, E.M. and Merrifield, R.B. (1989) Biological activities of des-His¹ [Glu⁹] glucagon amide, a glucagon antagonist. Peptides, 10: 1171-1177.

Unson, C.G., Macdonald, D., Ray, K., Durrah, T.L. and Merrifield, R.B. (1991) Position 9 replacement analogs of glucagon uncouple biological activity and receptor binding. J. Biol. Chem., 266: 2763-2766.

Unson, C.G., Wu, C., Fitzpatrick, K.J. and Merrifield, R.B. (1994) Multiple-site replacement analogs of glucagon. J. Biol. Chem., 269: 12548-12551.

Valverde, I., Morales, M., Clemente, F., López-Delgado, M., Delgado, E., Perea, A., and Villanueva-Penacarrillo, M.L. (1994) Glucagon-like peptide 1: a potent glycogenic hormone, FEBS lett., 349: 313-316.

Van Haastert, P.J.M., Dijkgraaf, P.A.M., Koniju, T.M., Abbad, E.G., Petridis, G. and Jastroff, B. (1983) Substrate specificity of cyclic nucleotide phosphodiesterase from beef heart and from Dictyoselium discoideum. Eur. J. Biochem. 131: 659-666.

Villanueva-Penacarrillo, M.L., Delgado, E., trapote, M.A., Alcantara, A., Clemente, F. and Valverde, I. (1993) Presence and characterization of GLP-I (7-36) amide receptors in the rat liver. Diabetologia, 36(Supp.1): A39, pp: 146.

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

Walker, J.C. and Oliver, D.J. (1986) Glycine decarboxylase multienzyme complex. Purification and partial characterization from pea leaf mitochondria. J. Biol. Chem., 261: 2214-2221.

Weir, G.C., Mojsov, S., Hendrick, G.K. and Hanener, J.F. (1989) Glucagonlike peptide I (7-37) actions on endocrine pancreas. Diabetes, 38: 338-342.

Wheeler, M.B., Lu, M., Dillon, J.S., Leng, X.H., Chen, C. and Boyd, A.E. (1993) Functional expression of the rat glucagon-like peptide-1 receptor, evidence for coupling to both adenylyl cyclase and phospholipase C. *Endocrinology*, 133: 57-62.

Whitton, P.D., Rodrigues, L.M. and Hems D.A. (1978) Stimulation by vasopressin, angiotensin and oxytocin of gluconeogenesis in hepatocyte suspensions. *Biochem. J.*, 176: 893-898.

Yoshida, T. and Kikuchi, G. (1972) Comparative study on major pathways of glycine and serine catabolism in vertebrate livers. *J. Biochem.*, 72: 1503-1516.

Yoshida, T. and Kikuchi, G. (1973) Major pathways of serine and glycine catabolism in various organs of the rat and cock. *J. Biochem.*, 73: 1013-1022.

Yu, Y.M., Yang, R.D., Matthews, D.E., Wen, Z.M., Burke, J.F., Bier, D.M. and Young, V.R. (1985) Quantitative aspects of glycine and alanine nitrogen metabolism in postabsorptive young men: Effects of level of nitrogen and dispensible amino acid intake. *J. Nutr.*, 115: 399-410.

* * ** * *



