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Characterization of the Hypersensitive Response of Glycogen Phosphorylase to Catecholamine Stimulation in Primary Culture Diabetic Cardiomyocytes: A Thesis

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CHARACTERIZATION OF THE HYPERSENSITIVE RESPONSE OF GLYCOGEN PHOSPHORYLASE TO CATECHOLAMINE STIMULATION IN PRIMARY CULTURE DIABETIC CARDIOMYOCYTES

A THESIS PRESENTED

BY

JO ANN BUCZEK-THOMAS

Submitted to the Faculty of the

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August 1992

DEDICATION

This thesis is dedicated with love to my mother, Joan Buczek and to the memory of my father, Joseph Buczek.

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There are many people who have provided their time and assistance throughout my graduate education to whom I am truly grateful.

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ABSTRACT

The primary goal of my thesis research was to characterize the basis for the hypersensitive response of glycogen phosphorylase to catecholamine stimulation in primary culture diabetic cardiomyocytes. Toward this goal, I have investigated several key regulatory sites in this signaling pathway which could promote the hypersensitive activation of phosphorylase. Specifically, I investigated (1) which adrenergic receptors are involved in mediating the hypersensitive response of glycogen phosphorylase to epinephrine stimulation; (2) whether the presence of fatty acid metabolites affects phosphorylase activation; (3) whether the hypersensitive response of phosphorylase results from altered signal transduction through the β-adrenergic receptor system or from a

post-receptor defect; and (4) the potential role for phosphorylase kinase in mediating the hypersensitive response of phosphorylase to catecholamine stimulation.

The basis for adrenergic receptor mediation of the catecholamine-induced activation of glycogen phosphorylase was investigated in adult rat cardiomyocytes isolated from normal and alloxan-diabetic animals. Cells derived from diabetic animals exhibited a hypersensitive response to epinephrine stimulation which was apparent 3 hours after cell isolation and was further enhanced upon maintenance of the myocytes in culture for 24 hours. Normal cells initially lacked the hypersensitive response to epinephrine stimulation although upon maintenance of these cells in culture for 24 hours, the hypersensitive response was acquired *in vitro*. To assess alpha- and beta-adrenergic mediation of the response, normal and diabetic cardiomyocytes were incubated with propranolol, a β-receptor antagonist, prior to direct α_1 receptor stimulation with phenylephrine. Both normal and diabetic myocytes failed to undergo activation of phosphorylase in 3 or 24 hour cell cultures. In addition, the effects of epinephrine on phosphorylase activation were completely inhibited by propranolol whereas prazosin, an α-receptor antagonist, was unsuccessful. This data suggests that the hypersensitive response of glycogen phosphorylase in normal and diabetic cardiomyocytes is solely mediated through β-adrenergic receptor activation.

Since the accumulation of various fatty acid metabolites can affect certain enzymes and signal transduction pathways within the cell, the potential effect of various fatty acid metabolites on phosphorylase activation was investigated. To determine the potential effects of fatty acid metabolites on phosphorylase

activation in cultured cardiomyocytes, normal and alloxan-diabetic cells were incubated with either carnitine or palmitoylcarnitine prior to stimulation with epinephrine. Pretreatment of cardiomyocytes with or without carnitine or palmitoylcarnitine for 3 or 24 hours before epinephrine stimulation failed to alter phosphorylase activation. The addition of exogenous carnitine in the absence and presence of insulin was also unsuccessful in attenuating the hypersensitive phosphorylase activation response in 3 and 24 hour, normal and alloxan-diabetic derived cardiomyocytes. To determine if carnitine palmitoyltransferase 1 (CPT-1) activity was responsible for the hypersensitive response of phosphorylase in the diabetic myocytes, both normal and diabetic myocytes were maintained for 3 and 24 hours in the absence and presence of etomoxir, a CPT-1 inhibitor. Subsequent activation of phosphorylase by epinephrine in normal and diabetic myocytes was unaltered in the presence of etomoxir. Collectively, these data fail to support a critical role for fatty acid metabolite involvement in the hypersensitive activation of glycogen phosphorylase in acute, alloxan-diabetic cardiomyocytes.

To assess potential G-protein involvement in the response, normal and diabetic-derived myocytes were incubated with either cholera or pertussis toxin prior to hormonal stimulation. Pretreatment of cardiomyocytes with cholera toxin resulted in a potentiated response to epinephrine stimulation whereas pertussis toxin did not affect the activation of this signaling pathway. To determine if the enhanced response of phosphorylase activation resulted from an alteration in adenylyl cyclase activation, the cells were challenged with forskolin. After 3 hours in primary culture, diabetic cardiomyocytes exhibited a hypersensitive response to forskolin stimulation relative to normal cells. However, after 24 hours in culture, both normal and diabetic myocytes responded identically to forskolin challenge.

The present data suggest that a cholera toxin sensitive G-protein mediates the hypersensitive response of glycogen phosphorylase to catecholamine stimulation in diabetic cardiomyocytes. This response, which is present in alloxan-diabetic cells, and is induced *in vitro* in normal cardiomyocytes, is primarily due to a defect at a post-receptor site.

To assess the role of phosphorylase kinase in the hypersensitive activation of glycogen phosphorylase in the diabetic heart, phosphorylase kinase activity was measured initially in perfused hearts (to optimize the assay parameters) and subsequently in primary culture cardiomyocytes. Results from these experiments demonstrate that the present method for measuring phosphorylase kinase activity is a reliable indicator of the enzyme's activity in the heart, although the assay conditions must be further optimized before this system can be applied to the measurement of phosphorylase kinase activity in primary cultured cardiomyocytes.

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ABBREVIATIONS

α-receptor	alpha adrenergic receptor
A-kinase	cAMP dependent protein kinase
AMP or 5'AMP	adenosine monophosphate
ATP	adenosine triphosphate
β-receptor or B-AR	beta adrenergic receptor
BSA	bovine serum albumin
cAMP	adenosine 3',5'-cyclic monophosphate
CoA	coenzyme A
CPT	carnitine palmitoyltransferase
DMSO	dimethyl sulfoxide
EC_{50}	half maximal concentration
EDTA	ethylenediaminetetraacetic acid
EPI	epinephrine
ETO	etomoxir
Gi	inhibitory guanine-nucleotide regulatory protein
Gs	stimulatory guanine-nucleotide regulatory protein
G-protein	guanine-nucleotide-binding regulatory protein
IMP	inosine monophosphate
Km	Michaelis constant
MEM	minimal essential medium

PGE_1	prostaglandin E_1
PMSF	phenylmethylsulfonyl fluoride
P'tase	protein phosphatase
PKI	cAMP-dependent protein kinase inhibitor
S.E.M.	standard error of the mean
T_3	triiodothyronine
TCA	trichloroacetic acid
Tris	tris (hydroxymethyl) aminomethane
Vmax	maximal velocity

CHAPTER I

Introduction

Phosphorylase (α-1,4-gulcan: orthophosphate glycosyl transferase, E.C. 2.4.1.1.), catalyzes the initial step in the intracellular degradation of glycogen by liberating glucose-1-phosphate from the glycogen particle (**Figure 1**) (46). Phosphorylase exists in the cell in two forms: phosphorylase b, which is essentially nonactivated *in vivo* and phosphorylase a, which is physiologically active (30). The active enzyme is a dimer composed of two identical 97-kdal subunits (46). Phosphorylase, as with other enzymes involved in glycogen metabolism, is regulated by both allosteric effectors and reversible serine phosphorylation, but of the two, phosphorylation is the dominant regulatory mechanism (9,47).

Phosphorylase b, the dephosphorylated form of the enzyme, is active only in the presence of high concentrations of AMP, which acts allosterically (46). AMP and, to a lesser extent, IMP, bind to the nucleoside binding site of the enzyme to alter its conformation and enhance its activity (9,30). ATP acts as a negative allosteric effector by competing with AMP, whereas, glucose-6-phosphate also inhibits phosphorylase b by binding at the same site (46). In contrast, phosphorylase a is fully active regardless of the levels of AMP, ATP, and glucose-6-phosphate (30).

Phosphorylase b may be converted to phosphorylase a upon phosphorylation (69,82). In response to nervous or hormonal stimulation, phosphorylase b is phosphorylated at a single position in the amino-terminus, serine-14, by phosphorylase kinase as part of the cAMP cascade (47,82). Activation by phosphorylation is reversed by the reciprocal action of phosphorylase phosphatase, a type 1 protein phosphatase (9,21,62). The rate of cellular glycogenolysis is directly dependent on the balance of opposing enzymatic activities which are responsible for maintaining phosphorylase in the a form.

The role of catecholamines in cardiac function has been well documented. In 1965, it was reported that epinephrine caused a rapid rise in cAMP production, enhanced both chronotropic and inotropic response and promoted the conversion of phosphorylase from the b to the a form in the perfused rat heart (74). With time, the cascading sequence of events by which epinephrine induced the activation of phosphorylase was described (**Figure 2**).

Activation of glycogen phosphorylase by epinephrine requires the coupling of cell surface receptors to the adenylyl cyclase system by receptor-linked G-proteins (4,15). For hormones which utilize cAMP as an intracellular messenger, two groups of G-proteins, Gs and Gi mediate the receptor-dependent stimulation or inhibition of adenylyl cyclase activity respectively (34,81). Stimulation of adenylyl cyclase results in increased intracellular cAMP levels which then activates cAMP-dependent protein kinase (A-kinase) (73). A-kinase then phosphorylates and activates phosphorylase kinase which catalyzes the conversion of phosphorylase from the b to the a form (22,71,83).

In diabetic or severely insulin deficient rats, cardiac glycogen phosphorylase activation, in response to epinephrine challenge is altered. When hearts from alloxan-diabetic rats were perfused for periods up to 10 minutes with epinephrine, activation of phosphorylase in diabetic hearts was significantly greater at every time point and at every epinephrine concentration than the normal response to the hormone (64). The diabetic response to epinephrine was termed "hypersensitive" (63,64). In the diabetic heart, protein kinase activity and cAMP levels were not shown to be significantly different than the normal response to epinephrine stimulation (1,64). In these hearts, phosphorylase phosphatase activity was found not to be altered by diabetes or epinephrine challenge whereas, phosphorylase kinase activation in the diabetic hearts was double the normal response to the hormone (64). Subsequent studies utilizing isolated perfused hearts from alloxan-diabetic animals showed that upon perfusion of the hearts with sub physiological calcium concentrations, approximately one-third of the normal perfusate calcium concentration, partially reversed the diabetes-related hypersensitivity of phosphorylase activation by epinephrine (63). What these data collectively suggested is that the hypersensitivity of phosphorylase activation in the diabetic was due to enhanced sensitivity of phosphorylase kinase activation by a factor or factors beyond the protein kinase level, possibly due to an increase in intracellular free calcium concentrations.

The majority of these types of studies were carried out using isolated perfused hearts which limit the information which can be obtained. The perfused heart preparation is limited as to the length of the perfusion time due to metabolic and hemodynamic failure (89). Upon establishment of a primary cell culture

system, further studies addressing the response of phosphorylase in the diabetic heart could be undertaken (89). Myocytes isolated from hearts of adult rats offer a number of distinct advantages over other heart preparations. Since this preparation results in a high yield of a single cell type, isolated myocytes are ideal preparations for biochemical and *in vitro* pharmacological studies (28,89). Primary culture techniques using laminin coated culture dishes significantly enhanced the plating efficiency of the preparation and enabled the cardiomyocytes to be maintained under tightly controlled conditions (11,89). Since primary culture cells are viable for several days or even weeks, this system lends itself to the study of the mechanisms involved in the diabetes-related hypersensitivity of phosphorylase (89). The initial studies using this system showed that cells derived from alloxan-diabetic hearts exhibited a hypersensitive phosphorylase activation response after placement into primary culture which was similar to that demonstrated with the perfused heart preparation (89). Upon maintenance of normal and alloxan-diabetic derived cardiomyocytes in culture for 24 hours, it was shown that diabetic myocytes were further sensitized to stimulation with epinephrine and the effect was induced *in vitro* to the same extent in normal cardiomyocytes (11). The physiological basis for the hypersensitive response in the diabetic and the *in vitro* acquisition of the response in the normal myocytes remains unknown.

The focus of my dissertation has been geared toward characterizing the molecular basis for the hypersensitive response of glycogen phosphorylase to epinephrine stimulation in primary culture, alloxan-diabetic cardiomyocytes. Toward this goal, I have addressed the following questions:

1. Which adrenergic receptors are involved in mediating the hypersensitive response of glycogen phosphorylase to epinephrine stimulation?

2. Does the accumulation of fatty acid metabolites affect phosphorylase activation in acute, diabetic-derived cardiomyocytes?

3. Does the hypersensitive response of glycogen phosphorylase activation to catecholamine stimulation result from altered signal transduction through the β-adrenergic receptor system or from a post-receptor defect?

4. Is the hypersensitive response of phosphorylase due to a defect at the level of phosphorylase kinase?

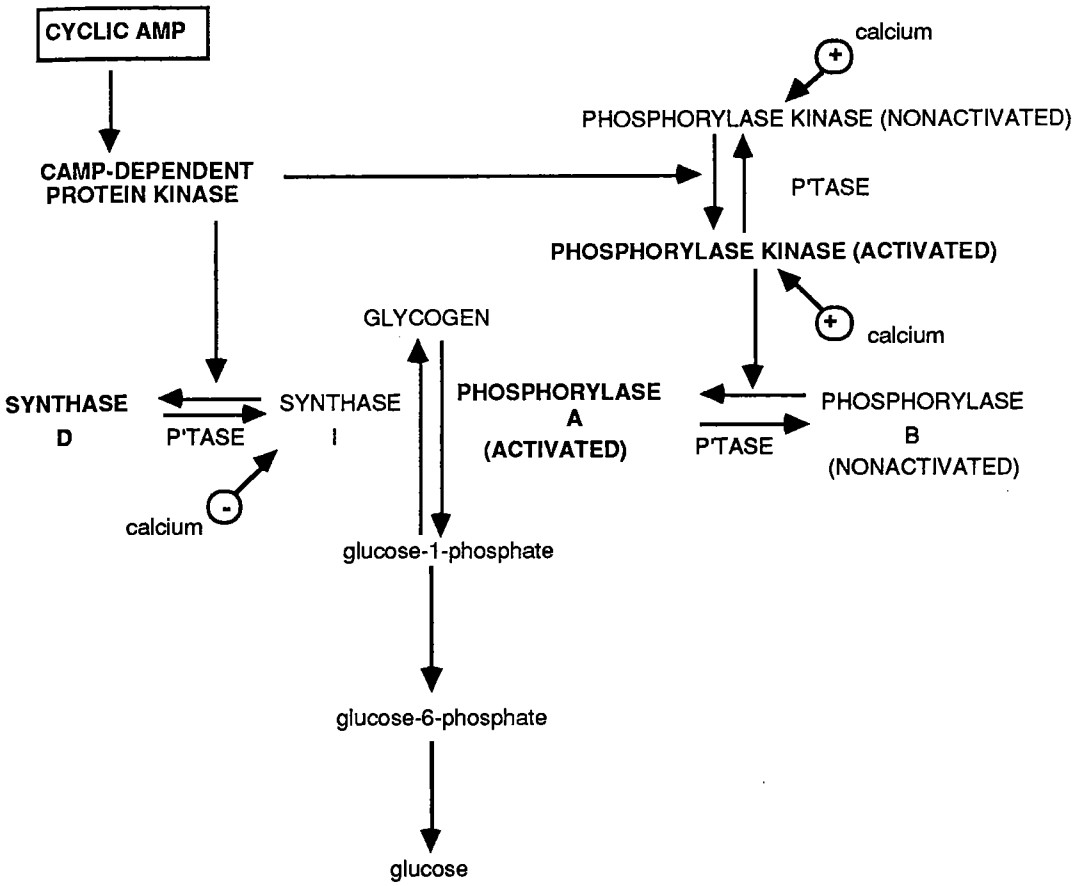

Figure 1. **Overview of glycogen metabolism.**

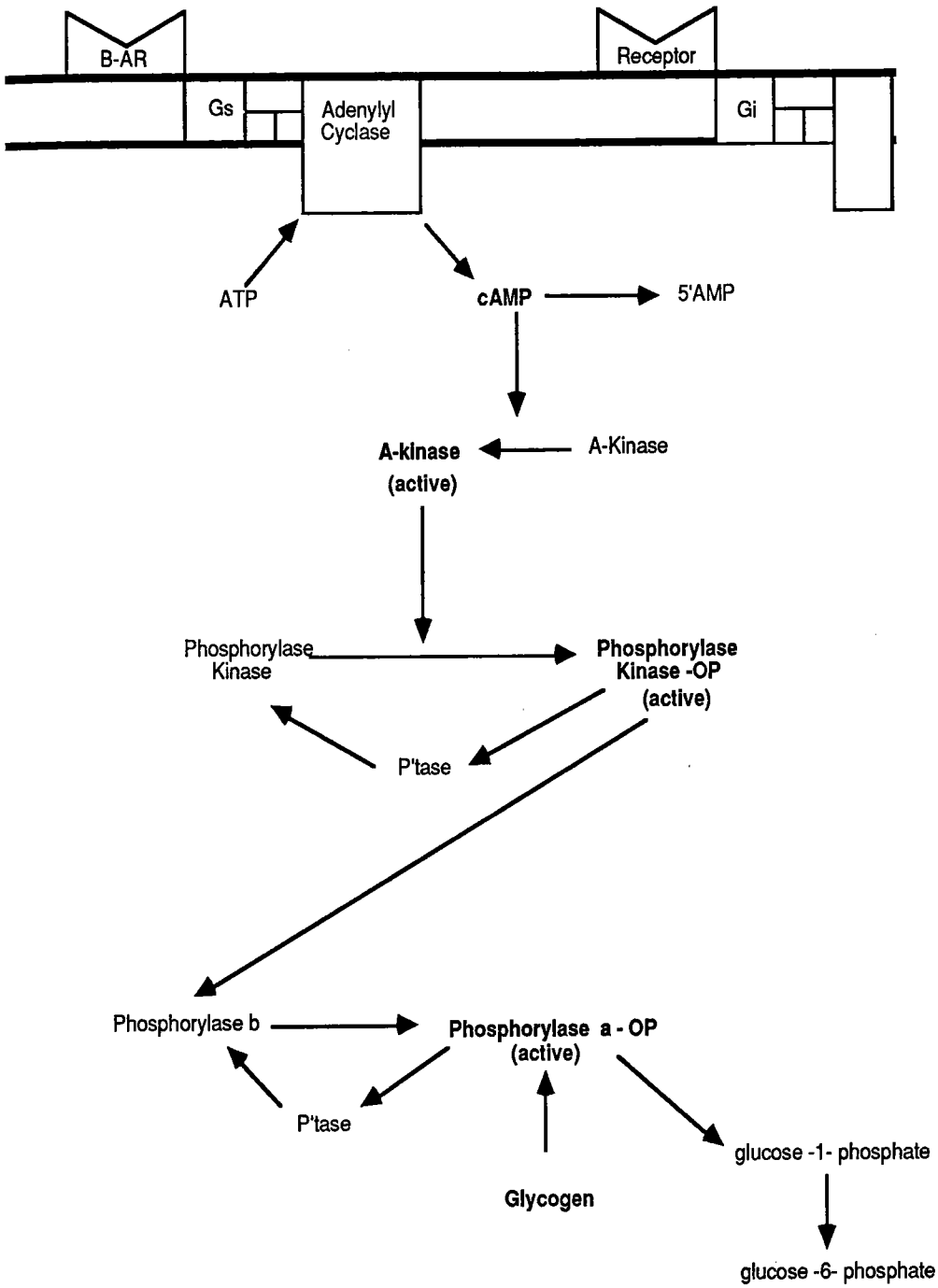

Figure 2. **Pathway for epinephrine-induced activation of glycogen phosphorylase.**

CHAPTER II

Materials and Methods

A. Reagents.

[^{14}C]glucose-1-phosphate, [^{35}S]methionine protein labeling mix and [γ-^{32}P]ATP were purchased from New England Nuclear. Epinephrine, propranolol, prazosin, carnitine, palmitoylcarnitine, forskolin, cholera and pertussis toxins, phosphorylase kinase and crystalline bovine serum albumin were purchased from Sigma. Phosphorylase b was purchased from Sigma, Boehringer Mannheim and was generously provided by Dr. James Dobson. Etomoxir was generously provided by Dr. H.P.O. Wolf of Byk Gulden Pharmazeutika (Konstanz, Germany). Laminin was obtained from Collaborative Research and Boehringer Mannheim. Collagenase and hyaluronidase were purchased from Worthington. All other chemicals were reagent grade products obtained from commercial sources.

B. Animals.

Male, albino, Sprague-Dawley CD outbred rats weighing between 175 and 250 grams were used for all experiments. Diabetes was induced by injection of alloxan (60 mg/kg) into the tail veins of animals which had been previously fasted overnight. Animals were used 3 to 5 days after alloxan injection and diabetes was diagnosed the day of perfusion using Ames Keto-Diastix (positive maximum glucose and ketone bodies in urine).

C. Preparation of Cardiomyocytes.

Cardiomyocytes were prepared according to the method described by Wolleben *et al.* (89). Briefly, hearts were perfused through the aorta in a retrograde fashion with Krebs-Henseleit bicarbonate buffer containing 5 mM glucose and 2.5 mM calcium. All hearts were equilibrated in this manner and then briefly switched to a second reservoir containing the same buffer without calcium to stop contraction of the hearts. The hearts were then perfused with Krebs-Henseleit buffer containing 50 μM calcium, 0.1% bovine serum albumin (BSA), 312 U/ml hyaluronidase, and 0.1% collagenase. After this final perfusion, the ventricles were trimmed free of atria and incubated in Krebs-Henseleit buffer containing 50 μM calcium, 0.2% BSA, 312 U/ml hyaluronidase and 0.1% collagenase in a 37°C water bath with gentle shaking. At 10 minute intervals, the enzyme solution was aspirated and replaced, and the incubation was continued until the tissue became flaccid. The ventricular tissue was dissociated by vigorous shaking for 10 minutes at 37°C. The resulting cell suspensions were filtered through nylon mesh and washed with Krebs-Henseleit buffer containing 100 μM calcium and 0.5% BSA. The cells were allowed to pellet by gravity and were washed twice in the same buffer prior to resuspension in minimal essential media (MEM) containing Earles' salts, 26 mM sodium bicarbonate, 5 mM creatine, 20 mM Hepes, 100 U/ml Penicillin G , 100 μg/ml streptomycin and 1.8 mM calcium (supplemented MEM). The myocytes were then seeded onto 60 mm laminin coated (20 μg/ml), Falcon plastic culture dishes containing 2 ml of the same media. The cells were maintained in a 95% air:5% CO_2 incubator for the duration of the experiment. All cells were allowed to equilibrate for 2 hours prior

to washing and refeeding with the same media containing 0.2 mM palmitate and 0.24% albumin.

For the adrenergic receptor studies, freshly isolated cells (2 hours in culture), were equilibrated for one additional hour in the absence or presence of adrenergic antagonists prior to hormonal challenge. Cells were maintained in culture for 24 hours in supplemented MEM containing 0.2 mM palmitate and 0.24% albumin in the absence of antagonists prior to refeeding with the same media, with or without antagonists. As with 3 hour cells, 24 hour cells were allowed to equilibrate for 1 hour after refeeding prior to hormonal challenge.

For the fatty acid metabolite studies, cells were maintained in primary culture for 2 hours prior to washing and refeeding with medium containing 0.24% albumin (without palmitate) and the appropriate concentration of palmitoylcarnitine, carnitine and/or insulin for 1 or 22 additional hours prior to epinephrine challenge. For the studies with etomoxir, cardiomyocytes were maintained in primary culture for 2 hours prior to washing and refeeding with medium containing 0.24% albumin (without palmitate) and the appropriate concentration of etomoxir for the appropriate time prior to stimulation with epinephrine.

For the G-protein studies, cells were maintained in primary culture for 2 hours and were equilibrated in the absence or presence of 25 μg/ml cholera toxin or 1 μg/ml pertussis toxin for 1 hour prior to stimulation with epinephrine. Three hour cells for the forskolin dose response studies were equilibrated for 1 additional hour in supplemented MEM containing 0.2 mM palmitate and 0.24% BSA and 24 hour cells were maintained in culture overnight in the same medium.

Forskolin stocks (10 mM) were prepared fresh in dimethyl sulfoxide (DMSO) and serial dilutions were made using 0.9% sterile saline.

For cells isolated for the phosphorylase kinase studies, the cells were maintained in primary culture for 2 hours prior to washing and refeeding with medium containing 0.2 mM palmitate and 0.24% albumin. The cardiomyocytes were maintained in primary culture for one additional hour (3 hours total) or overnight (24 hours total) prior to epinephrine challenge. After agonist exposure for the appropriate time, all myocytes were washed with buffer containing 50 mM tris (pH 7.8), and 5 mM EDTA prior to freezing by floating the culture dish on liquid nitrogen and storing at -70^{o}C.

D. Phosphorylase Assay.

Frozen cells were thawed on ice and the cells scraped from the dish using a rubber policeman. Myocytes were washed with ice cold homogenization buffer containing 50 mM tris (pH 7.8), 5 mM EDTA, 50 mM sodium fluoride and 1 mg/ml oyster glycogen. The resulting suspensions were sonicated for 30 seconds prior to microcentrifugation for 5 minutes. Cell supernatants were assayed for phosphorylase activity in the absence and presence of AMP, based on the method of Gilboe *et al.* which measures [^{14}C]glucose-1-phosphate incorporation into glycogen (32). Phosphorylase activity is expressed as the ratio of AMP independent (phosphorylase a activity) to total activity assayed in the presence of AMP (phosphorylase a + b activity) to determine " % phosphorylase a".

Glycogen phosphorylase activity may be assayed in either the direction of glycogen degradation or glycogen synthesis. In the forward reaction, toward

glycogen degradation, phosphorylase activity is measured based on glucose-1-phosphate release from glycogen, in the presence of inorganic phosphate (12,38). Although this assay method is more physiologically relevant for these studies, there are several drawbacks (*i.e.* low sensitivity and high background) which render this protocol inappropriate for use with primary culture cardiomyocytes (32,38). The preferred method for monitoring changes in phosphorylase activity was to measure the reverse reaction, toward glycogen synthesis at decreased pH. Under slightly more acidic conditions (pH 6.1), the reaction equilibrium favors phosphorylase activity in the direction of glycogen synthesis (32,33). In addition, phosphoglucomutase, the enzyme which catalyzes the conversion of glucose-1-phosphate to glucose-6-phosphate and is present in cell extracts, is inhibited at this pH to yield more accurate results (33). Since the reverse reaction toward glycogen synthesis monitors [^{14}C]glucose-1-phosphate incorporation into glycogen, the specific activity of glucose-1-phosphate may be increased to enhance the assay's sensitivity and to optimize the system for use in microassays or with various heart preparations (32).

E. Metabolic Cell Radiolabeling.

Before labeling, normal and diabetic isolated cardiomyocytes were incubated for 2 hours prior to washing and refeeding. The medium was replaced with fresh supplemented MEM containing 0.2 mM palmitate, 0.24% albumin and 50 μCi/ml [^{35}S]methionine protein labeling mix, with or without cycloheximide, and the cells were incubated for an additional 2 hours. After labeling, the cells were rinsed twice with ice cold buffer containing 50 mM tris (pH 7.8) and 5 mM

EDTA and frozen by floating the plate on liquid nitrogen. Dishes were stored at -70°C before analysis.

F. Total Protein Synthesis Determination.

Using a rubber policeman, frozen cardiomyocytes were scraped from culture dishes in lysis buffer containing 50 mM tris (pH 7.4), 5 mM EDTA, 100 mM sodium fluoride, 1% NP-40, 1 mM PMSF, 1 mM benzamidine, 1 µg/ml leupeptin and 1 µg/ml pepstatin. The suspensions were homogenized for 12 seconds with a Polytron homogenizer and were centrifuged at 10,000 x g for 15 minutes. The supernatants were assayed for protein content. Aliquots containing 10 µg of sample protein were precipitated onto a nitrocellulose filter (Schleicher and Schuell) with ice cold 10% trichloroacetic acid (TCA) in a BRL dot blot apparatus. Each sample was washed twice with an additional 250 µl of 10% TCA and individual spots were cut and counted by liquid scintillation methods using Optifluor to determine [^{35}S]methionine incorporated into protein.

G. Phosphorylase Kinase Assay.

Phosphorylase kinase activity was assayed based on the method of McCullough and Walsh (58) as modified in (2) which measures the incorporation of ^{32}P from [γ-^{32}P]ATP into phosphorylase b. Briefly, rat hearts were perfused with glucose-free, Krebs-Henseleit buffer containing 2.5 mM calcium for 30 minutes to deplete the endogenous glycogen stores. Control hearts (- Epi) were freeze clamped using Wollenberger tongs pre-cooled in liquid nitrogen while the

remaining hearts were perfused for an additional 5 minutes with the same buffer containing 10 μM epinephrine (+ Epi) before freezing.

Frozen hearts were individually powdered using a mortar and pestle and 150 mg of heart powder was suspended in approximately 2 volumes of homogenization buffer containing 30 mM tris (pH 7.5), 30 mM potassium chloride, 5 mM EDTA, 100 mM sodium fluoride, 1 mM PMSF, 1 mM benzamidine and 1 μg/ml leupeptin and pepstatin. The heart extracts were then sonicated for 60 seconds and microcentrifuged. The resulting supernatants were diluted with an equal volume of dilution buffer which contained 30 mM glycerophosphate (pH 6.8), 5 mM EDTA, 100 mM sodium fluoride and 45 mM 2-mercaptoethanol. The reaction was initiated by adding 10 μl of diluted extract or buffer ($0^{o}C$) to 50 μl of reaction mixture ($30^{o}C$) which consisted of 50 mM tris (pH 6.8), 53 mM glycerophosphate, 12 mM magnesium acetate, 20 μM calcium chloride, 12 mg/ml glycogen, 2 μg cAMP-dependent protein kinase inhibitor (PKI), 1 mM [γ-^{32}P]ATP (220 cpm/pmol), 18 mM 2-mercaptoethanol, with or without 6 mg/ml phosphorylase b. The reaction mixture was routinely incubated at $30^{o}C$ for 2 minutes prior to the addition of sample to avoid cold inactivation of phosphorylase b (33,72). The reaction was terminated after 10 minutes by spotting 40 μl of the reaction mixture onto Whatman filter papers which were immediately immersed into an ice cold wash containing 10% trichloroacetic acid (TCA) and 50 mM potassium pyrophosphate. The filters were washed three more times with 10% TCA, 50 mM potassium pyrophosphate followed by one wash each in 50% ethanol and acetone. The dried filters were counted by liquid scintillation methods with Optifluor.

Isolated myocytes were assayed for phosphorylase kinase by scraping the cells from the plates using a rubber policeman and homogenization buffer. Cell suspensions were sonicated and microcentrifuged. 10 µl of the cell supernatant (0°C), without further dilution, was added to 50 µl reaction mixture (30°C) to initiate the reaction. The assay incubation time was extended to 60 minutes and the reaction was terminated as described above.

H. Protein Content Determination.

In conjunction with the phosphorylase a assays, cell supernatants were routinely assayed for protein content using the Bio Rad Protein Assay. When [^{35}S]methionine labeled cells were harvested, detergent-containing extracts were assayed for protein content by the BCA Protein Assay.

I. Data Computation and Expression.

Data are expressed as means plus one standard error on either side of the mean. EC_{50} values were calculated by non regression analysis using Graph Pad software. Statistical significance was determined by Student's t-test.

CHAPTER III

Characterization of Adrenergic Receptor Involvement in Glycogen Phosphorylase Activation

A. Introduction.

Glycogen phosphorylase plays a key role in the control of energy supply in the heart by regulating the rate of glycogen degradation (glycogenolysis). Phosphorylase, as with other enzymes involved in glycogen metabolism, is regulated on a minute to minute basis by various circulating hormones which stimulate or depress its activity based on their ability to promote phosphorylation or dephosphorylation the enzyme (13,19,42). The phosphorylated form of the enzyme, phosphorylase a, is physiologically active while the dephosphorylated form, phosphorylase b, is nonactivated *in vivo*. The rate of glycogenolysis is therefore dependent on the amount of phosphorylase which is present in the a form.

Epinephrine, a circulating cardiac catecholamine, has been shown to produce an activation of glycogen phosphorylase activity which is significantly greater in hearts derived from alloxan-diabetic and spontaneously diabetic BB/W animals than from normal animals (61,63,64). While the catecholamine-induced activation of cardiac phosphorylase has been shown to result from an increase in phosphorylase kinase activity, cAMP levels and protein kinase activity in response to epinephrine in diabetic hearts were shown not to be significantly different than the normal in response to the hormone (1,64). While the majority of

epinephrine's stimulatory effects are thought to be mediated through the activation of β-adrenergic receptors (25,78), recent reports have indicated that the actual number of surface β-adrenergic receptors in the diabetic rat heart decreases without altering the affinity of the receptor for the agonist (44,48,78). Since the hypersensitive response of diabetic glycogen phosphorylase to catecholamine stimulation does not appear to result from a simple change in receptor number or agonist affinity, and since epinephrine is capable of eliciting both α- and β-adrenergic responses in the heart, a potential role for α-adrenergic mediation of the hypersensitivity effect exists.

The purpose of the following study was to determine whether the hypersensitivity of glycogen phosphorylase activation by catecholamines in alloxan-diabetic hearts is mediated by an α- or β-adrenergic response. The following work demonstrates that the hypersensitive response to epinephrine stimulation in diabetic hearts occurs independently of α-adrenergic receptor activation and is mediated solely by the β-adrenergic receptor system.

B. Results.

Earlier studies carried out in this laboratory showed that cardiomyocytes in this primary culture system maintain their classic cardiac features such as longitudinal striations of fiber bundles and cross striations of the various bands (89). Plating of cardiomyocytes onto laminin-coated plastic culture dishes resulted in preparations with greater than 90% rod shaped, viable cells.

The data presented in **Figure 3** illustrate the dose dependent relationship for epinephrine-stimulated activation of glycogen phosphorylase in

cardiomyocytes from normal and alloxan-diabetic rats, 3 and 24 hours after isolation. Both normal and alloxan-diabetic 3 hour cells were sensitive to the effects of epinephrine over the concentration range of 1×10^{-7} M to 5.5×10^{-6} M and exhibited identical kinetic responses (data not shown). For normal myocytes, the half maximal response for phosphorylase activation occurred with 5.5×10^{-7} M $\pm$ 1.25 epinephrine whereas those from alloxan-diabetic rats were significantly ($p < 0.005$) more sensitive to epinephrine stimulation since the half maximal response was elicited with 2.4×10^{-7} M $\pm$ 1.05 epinephrine. After 3 hours in primary culture, the maximal response elicited to epinephrine challenge was 12% and 24% phosphorylase a for normal and alloxan-diabetic myocytes, respectively. Upon maintenance of these cells in culture for 24 hours prior to hormonal challenge, the effective epinephrine concentration range to which 24 hour myocytes were responsive underwent a significant ($p < 0.005$) leftward shift relative to those maintained in culture for 3 hours. After 24 hours, the half maximal concentration of epinephrine required for phosphorylase activation in myocytes from diabetic animals was an order of magnitude lower ($EC_{50} = 4.1 \times 10^{-8}$ M $\pm$ 1.10). In the normal, 24 hour cardiomyocytes, there was an *in vitro* induction of the hypersensitive response since the half maximal response was elicited with 4.4×10^{-8} M $\pm$ 1.17 epinephrine and is comparable to the diabetic myocyte, 24 hour response. After 24 hours in culture, the maximal response which was elicited to epinephrine stimulation was approximately 50% phosphorylase a for both normal and alloxan-diabetic derived cardiomyocytes. Similar dose response relationships were established for 3 and 24 hour, normal and alloxan-diabetic rat cardiomyocytes in response to stimulation with the β-agonist isoproterenol (data not shown). The mechanism by which this *in vitro*

hypersensitivity response is induced in normal cells and is further enhanced in diabetic cells in culture is unknown.

To determine if the hypersensitive response of phosphorylase to catecholamine stimulation was potentially mediated by an α-adrenergic effect, normal and diabetic cardiomyocytes were preincubated with propranolol, a β-adrenergic receptor antagonist, prior to challenge with phenylephrine, a selective α_1 receptor agonist. **Figure 4** clearly demonstrates that direct alpha adrenergic stimulation of normal and diabetic cardiomyocytes results in only minimal activation of phosphorylase. Unlike with epinephrine stimulation, there is no hypersensitive phosphorylase response elicited upon phenylephrine challenge.

To further address the potential role for alpha adrenergic mediation of the hypersensitive phosphorylase response to epinephrine, both normal and alloxan-diabetic cardiomyocytes were incubated with either prazosin, a selective α_1 receptor antagonist, or propranolol, a β_1/β_2 receptor antagonist, for 1 hour prior to epinephrine challenge. As illustrated in **Figures 5 and 6**, phosphorylase activation was completely inhibited when either normal or diabetic, 3 or 24 hour cells, were pretreated with propranolol. When 24 hour cardiomyocytes from alloxan-diabetic or normal hearts were preincubated with prazosin, epinephrine-stimulated activation of phosphorylase was attenuated by only 10%. There does not appear to be any involvement of muscarinic receptors in the hypersensitive response exhibited by the cells from alloxan-diabetic animals since the addition of 0.1 μM atropine to cultured normal and diabetic cardiomyocytes failed to prevent the hypersensitive phosphorylase response (data not shown). The hypersensitive response of phosphorylase is also independent of insulin or triiodothyronine (T_3) treatment. When normal and diabetic cardiomyocytes were

cultured for 24 hours in the absence or presence of insulin (0.1 μM), T_3 (75 μM) or insulin with T_3, the response of phosphorylase to epinephrine stimulation was unaltered (data not shown). In addition, induction of the hypersensitive response of phosphorylase to epinephrine stimulation also occurred independently of the presence of serum, BSA, palmitate and both eicosapentaenoic and docasahexaenoic acid, two fatty acids whose levels are known to be increased in the diabetic state (50,85). When applied exogenously to the culture medium, these agents were unsuccessful in attenuating the hypersensitive response of phosphorylase (data not shown). The hypersensitive response of glycogen phosphorylase to catecholamine stimulation also occurred independently of protein synthesis. When normal and alloxan-diabetic cardiomyocytes were cultured for 3 hours, **Figure 7**, or 24 hours, **Figure 8**, with 1 or 10 μM cycloheximide, the phosphorylase response to epinephrine challenge was unaltered. Incubation of primary cultured cardiomyocytes with cycloheximide was shown to decrease protein synthesis by approximately one-third in normal myocytes and one-half in diabetic cardiomyocytes as shown in **Table 1**. To what extent these agents may act in concert with other factors to abolish the response to adrenergic agonists is not known.

As depicted in earlier figures, diabetic cardiomyocytes exhibit an increased sensitivity to epinephrine after 3 hours in culture which was further enhanced after 24 hours. In normal cardiomyocytes, the hypersensitive activation of phosphorylase by epinephrine was acquired upon culturing the cells for 24 hours. These data suggest that the hypersensitive response of glycogen phosphorylase activation, which is present in myocytes derived from alloxan-diabetic rats and is

acquired *in vitro* by normal rat cardiomyocytes, is mediated solely by the β-adrenergic receptor pathway.

C. Discussion.

The present study shows that epinephrine-induced activation of glycogen phosphorylase in normal and alloxan-diabetic rat cardiomyocytes appears to be mediated solely through β-adrenergic receptor activation. When propranolol, a β-adrenergic receptor antagonist, was added to the cultured myocytes to inhibit this component of epinephrine's action, the observed hypersensitivity effect was abolished. Both prazosin and atropine failed to prevent the hypersensitive activation of phosphorylase as did cycloheximide, insulin and/or T_3. Isoproterenol was found to have effects similar to epinephrine in promoting phosphorylase activation whereas phenylephrine had limited ability to activate the enzyme. While earlier studies did suggest a potential role for α_1 adrenergic mediation of the hypersensitivity effect, the data presented in this report fails to support this hypothesis.

Since phosphorylase activity in both normal and alloxan-diabetic cells cultured for 3 or 24 hours is only minimally activated in response to direct α_1 stimulation with phenylephrine, a major role for α_1 mediation of the hypersensitivity effect is doubtful. In addition, prazosin, a selective α_1 receptor antagonist, had only limited success in blocking phosphorylase activation in all cell preparations which is in agreement with other published studies (17). The discrepancy between this work and earlier studies probably lies in the fact that the initial studies utilized the perfused heart system in which receptor blockade is

dependent at least in part, on perfusion efficacy. Also, lower concentrations of propranolol were used which may have been insufficient for complete β-blockade (64).

The possibility that the phosphorylase hypersensitivity effect may result as an isolation or primary culture artifact seems unlikely since the effect has been well documented utilizing other diabetic tissue preparations including the gravity flow perfused heart (64) and the isolated working heart (63). Since the hypersensitive response of phosphorylase is seen shortly after the isolation of diabetic cardiomyocytes (within 3 hours) and is absent initially in normal myocytes, the effect appears to be intrinsic to the diabetic state since both normal and diabetic cells are isolated and maintained in an identical fashion. It is only upon culturing of normal cardiomyocytes for 24 hours that the hypersensitivity of phosphorylase activation is acquired *in vitro* and is further enhanced in diabetic cardiomyocytes. The hypersensitive response of phosphorylase to epinephrine-stimulation does not appear to result from a change in the rate of protein synthesis as a consequence of placement into primary culture. When normal and diabetic-derived cardiomyocytes were maintained in culture for periods up to 72 hours, total protein synthesis as well as phosphorylase synthesis were unaffected by diabetes (77).

Catecholamines have been shown to alter calcium flux (63), and phosphorylase kinase, the enzyme responsible for converting phosphorylase to its active form, is subject to regulation, in part, by calcium (19). Several reports have documented abnormal calcium-metabolism in diabetic animals which, in turn, could result in the alteration of calcium-dependent cardiac metabolism or other calcium-sensitive systems (1,31,36,42,43,67). While the precise

mechanism for the hypersensitivity of phosphorylase activation in the diabetic heart, and the acquisition of this effect in cultured normal rat cardiomyocytes, is unknown, one likely possibility is that it may result from modified calcium homeostasis upon culturing of these cells. Changes in sarcoplasmic reticular calcium transport (31), pump function (43), or calcium channel flux (67) in the diabetic state have been documented and could all, individually, or in combination, act to increase intracellular calcium levels to a point at which glycogen phosphorylase kinase would respond in a hypersensitized fashion. Another mechanism which might mediate the hypersensitivity of phosphorylase activation is through altered signal transduction *via* the β-adrenergic receptor. Changes in receptor coupling or G protein interactions such as a decrease in Gi or an increase in Gs activity would elicit the respective changes in adenylyl cyclase activity which would be reflected in the activation of phosphorylase (73).

Since isolated rat cardiomyocytes contain primarily α_1 and β_1 receptors with no α_2 and few β_2 receptors present at the myocyte surface, an alternate means by which glycogen phosphorylase activation might be sensitized to epinephrine stimulation could result from an up-regulation of β_1-receptors at the cell surface during the time in which the cells are maintained in culture (3,14,17,87). This hypothesis seems unlikely since β-receptor density has been shown not to be significantly changed when isolated rat cardiomyocytes are maintained in culture for periods up to 10 days (57).

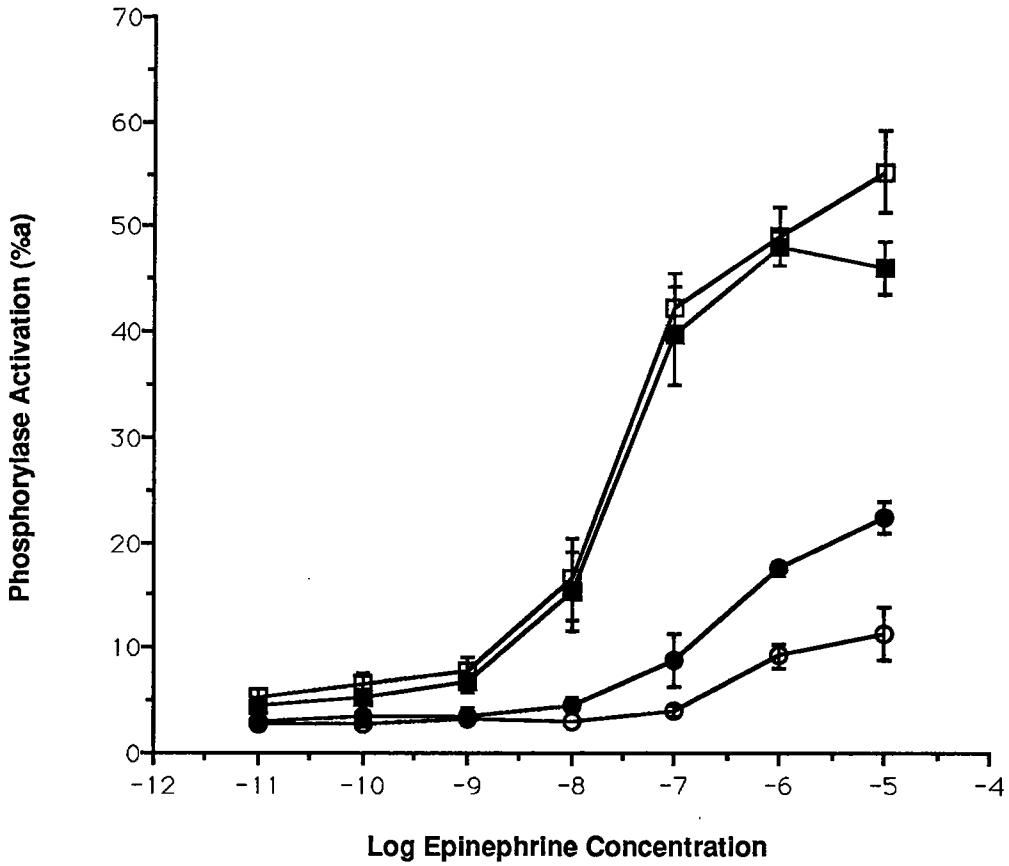

Figure 3. **Dose response relationship for epinephrine-stimulated phosphorylase activation in normal and alloxan-diabetic cardiomyocytes after 3 and 24 hours in culture.** Cardiomyocytes from normal and alloxan-diabetic animals were equilibrated in supplemented minimal essential media (MEM). Three hour normal (open circles) and diabetic cells (closed circles) were challenged with epinephrine for 10 minutes on the same day on which they were isolated whereas 24 hour normal (open squares) and diabetic (closed squares) cells were maintained in culture overnight prior to hormonal challenge. Cultured cells were assayed for phosphorylase activity. Plotted values are means ± S.E.M. where n=6.

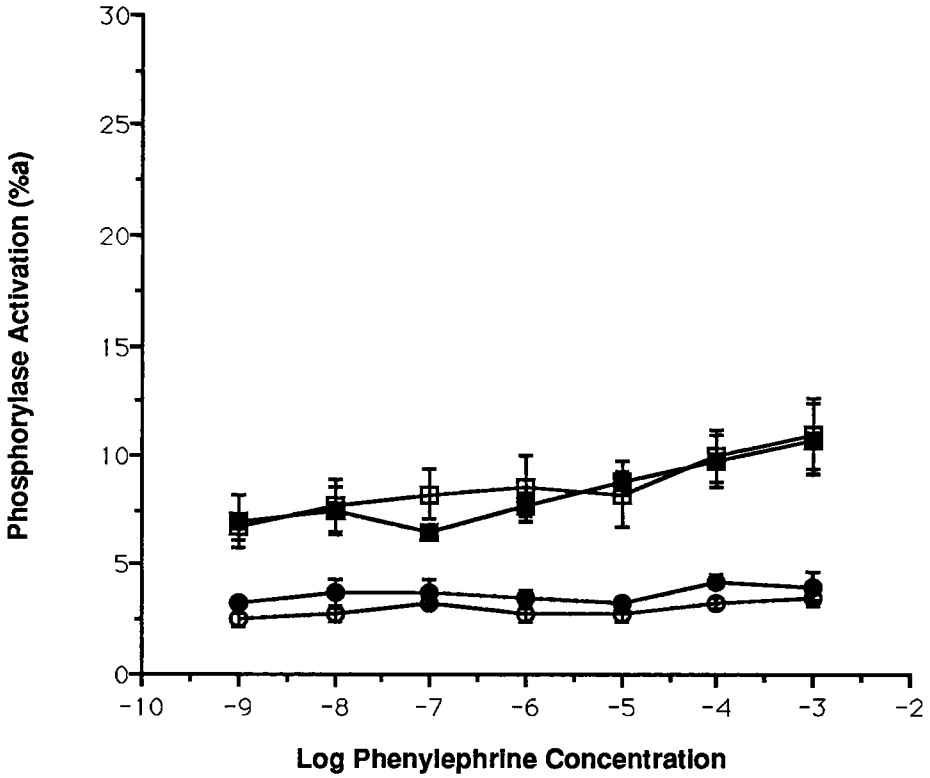

Figure 4. **Dose response relationship for phenylephrine-stimulated phosphorylase activation in normal and alloxan-diabetic cardiomyocytes after 3 and 24 hours in culture.** Cardiomyocytes from normal and alloxan-diabetic animals were isolated and equilibrated in supplemented MEM containing 10 μM propranolol for 1 hour prior to phenylephrine challenge. Three hour normal (open circles) and diabetic (closed circles) cells underwent agonist challenge for 10 minutes on the same day on which they were isolated while 24 hour normal (open squares) and diabetic cells (closed squares) were maintained in culture overnight prior to antagonist addition. Frozen myocytes were assayed for phosphorylase activity. Plotted values are means ± S.E.M. where n=4.

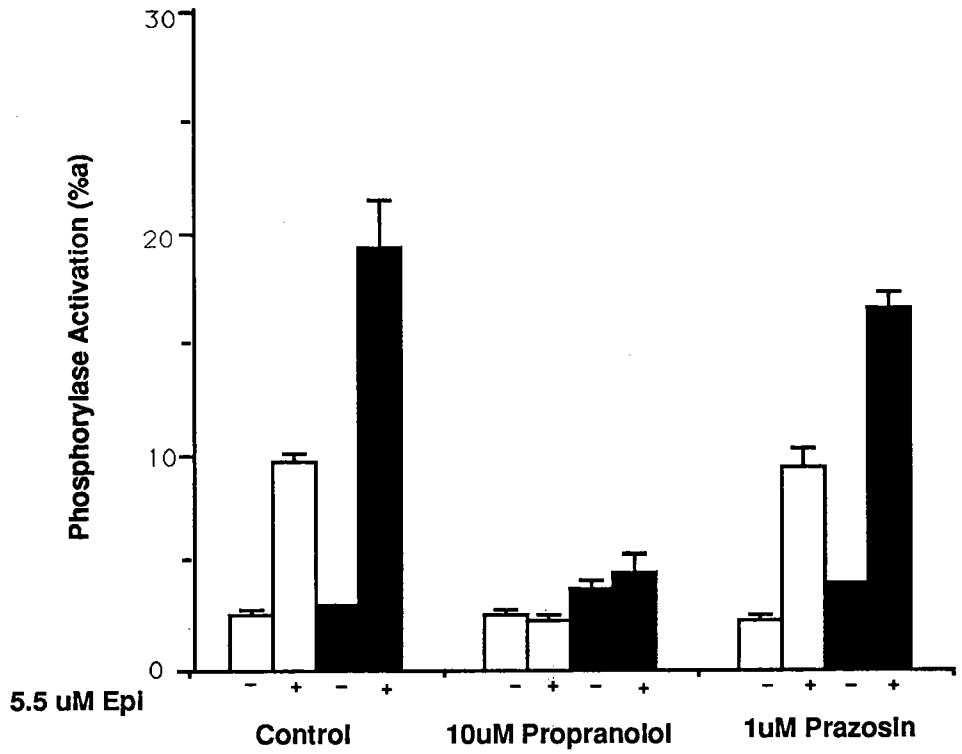

Figure 5. **Effect of adrenergic antagonists on epinephrine-stimulated activation of phosphorylase in normal and alloxan-diabetic cardiomyocytes after 3 hours in culture.** Cardiomyocytes from normal (open bars) and alloxan-diabetic animals (closed bars) were isolated and maintained in culture for 2 hours prior to equilibration with supplemented MEM containing either 10 μM propranolol or 1 μM prazosin for 1 hour. Three hour cardiomyocytes were then challenged with 5.5 μM epinephrine for 10 minutes and assayed for phosphorylase activity. Plotted values represent the mean ± S.E.M. where n=4.

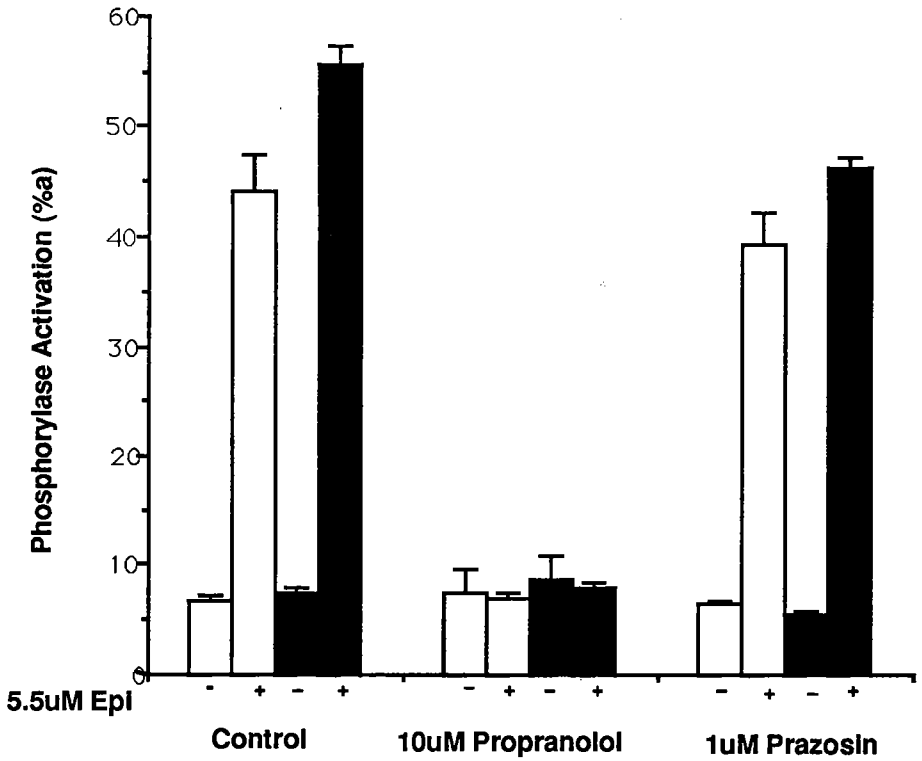

Figure 6. **Effect of adrenergic antagonists on epinephrine-stimulated phosphorylase activation in normal and alloxan-diabetic cardiomyocytes after 24 hours in culture.** Cardiomyocytes from normal (open bars) and alloxan-diabetic animals (closed bars) were isolated and maintained in culture overnight in the absence of antagonist. The cells were then equilibrated in supplemented MEM containing either 10 μM propranolol or 1 μM prazosin for 1 hour prior to agonist exposure. Myocytes were then challenged with 5.5 μM epinephrine for 10 minutes and assayed for phosphorylase activity. Plotted values express the mean ± S.E.M. where n=4.

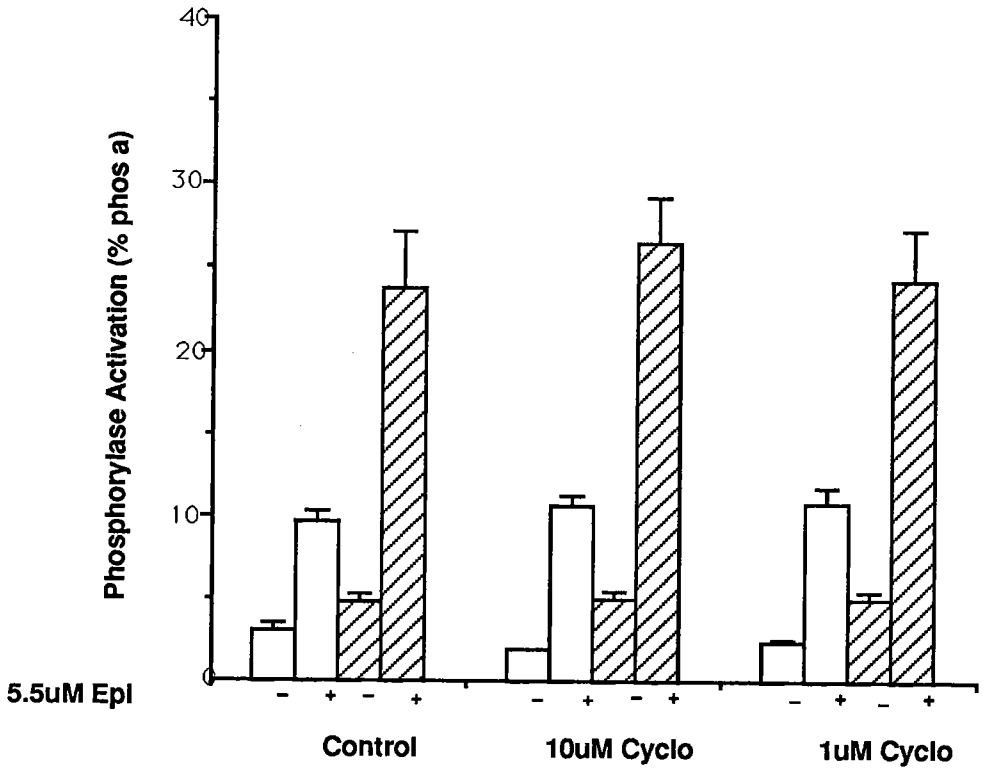

Figure 7. **Effect of protein synthesis inhibition by cycloheximide on epinephrine-stimulated phosphorylase activation in normal and alloxan-diabetic cardiomyocytes after 3 hours in primary culture.** Cardiomyocytes from normal and alloxan-diabetic animals were equilibrated in supplemented MEM with or without 1 or 10 μM cycloheximide. Three hour normal (open bars) and diabetic myocytes (hatched bars) were challenged with 5.5 μM epinephrine for 10 minutes and were subsequently assayed for phosphorylase activity. Plotted values are means ± S.E.M. where n = 6.

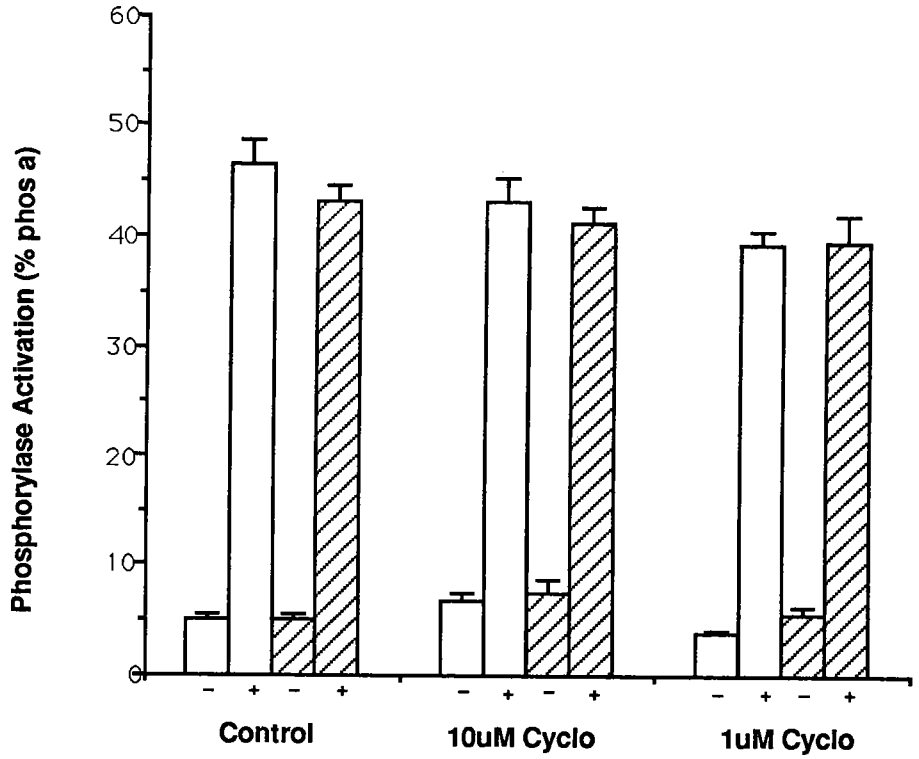

Figure 8. **Effect of protein synthesis inhibition by cycloheximide on epinephrine-stimulated phosphorylase activation in normal and alloxan-diabetic cardiomyocytes after 24 hours in culture.** Cardiomyocytes from normal and alloxan-diabetic animals were equilibrated in supplemented minimal essential medium with or without 1 or 10 μM cycloheximide. Twenty four normal (open bars) and diabetic myocytes (hatched bars) were challenged with 5.5 μM epinephrine for 10 minutes and were subsequently assayed for phosphorylase activity. Plotted values are means ± S.E.M. where n = 6.

Table 1

Effect of cycloheximide on [^{35}S]methionine incorporation in normal and alloxan-diabetic cultured cardiomyocytes

	Normal (% control)	**Diabetic** (% control)
Nontreated Myocytes	100	100
10μM Cycloheximide	68.0 ± 6.6	55.8 ± 4.4
1μM Cycloheximide	62.2 ± 8.7	55.3 ± 5.9

Isolated cardiomyocytes were cultured for 2 hours in medium containing [^{35}S]methionine protein labeling mix (50 μCi/ml) with or without cycloheximide. [^{35}S]methionine incorporation into acid precipitable protein was quantified after incubating the myocytes with the labeled amino acid. The data shown are the mean ± S.E.M. where n = 4.

CHAPTER IV

Effect of Fatty Acid Metabolites on Phosphorylase Activation

A. Introduction.

The onset of diabetes dramatically alters myocardial energy metabolism. In the heart, plasma free fatty acids are the principal substrate from which the myocardium derives its energy. In normal hearts, approximately 60% of the total energy for metabolism is supplied by the oxidation of fatty acids, while glycolysis provides approximately 30% (52). In the diabetic heart, the oxidation of fatty acids may account for greater than 90% of the total energy produced (52).

In fatty acid metabolism, carnitine plays an essential role by enabling long chain fatty acid moieties to be shuttled across the inner mitochondrial membrane into the mitochondrial matrix where β-oxidation occurs (**Figure 9**) (6,8,75). The fatty acids are first activated upon thioester linkage between the carbonyl group of the fatty acid and the sulfhydryl group of coenzyme A (CoA) to form acyl CoA. The acyl group is then transferred from the sulfur atom of CoA to carnitine *via* carnitine palmitoyltransferase 1 (CPT-1) to form acylcarnitine and regenerate free CoA. Newly formed acylcarnitine is transferred across the membrane by a translocase. The acyl group is then transferred back to CoA on the matrix side of the membrane *via* CPT-2. Free carnitine is then returned to the cytosolic side of the membrane by the translocase in exchange for the incoming acylcarnitine.

It has been shown in diabetic hearts that accompanying the increased utilization of fatty acids is a significant increase in the metabolites which are

associated with fatty acid metabolism (29,52). The accumulation of fatty acid metabolites is known to have adverse effects on the heart since these compounds have been shown to affect critical enzyme systems (55), to alter membrane integrity (35,75), and to affect certain signal transduction pathways (49). The focus of the present study was to assess the potential effects of fatty acid metabolites on glycogen phosphorylase activation in normal and acute, alloxan-diabetic, primary culture cardiomyocytes. These studies demonstrate that the hypersensitive response of phosphorylase to epinephrine in normal and acute diabetic myocytes occurs independently of the presence of various fatty acid metabolites.

B. Results.

The rationale behind this series of experiments was that upon the addition of acylcarnitine, in this case palmitoylcarnitine, to cultured normal and alloxan-diabetic cardiomyocytes, one would expect to see a potentiated phosphorylase activation response to epinephrine if the presence of acylcarnitine was affecting the enzyme. Similarly, if carnitine was rate limiting for acylcarnitine transport into the matrix, after supplementation of the cultures with exogenous carnitine, one would expect to see an attenuated phosphorylase activation response. The data presented in **Figures 10 A and 10 B** illustrate the effect of palmitoylcarnitine and carnitine on epinephrine-stimulated phosphorylase activation in normal (**Figure 10 A**) and acute, alloxan-diabetic cardiomyocytes (**Figure 10 B**) after 3 hours in primary culture. Supplementation of the culture medium with either 10 μM palmitoylcarnitine or 1 mM carnitine failed to alter the phosphorylase activation

response to epinephrine in both normal and alloxan-diabetic derived cardiomyocytes. The response of alloxan-diabetic myocytes with or without palmitoylcarnitine or carnitine, was hypersensitive with respect to the normal myocyte response and is reflected by the change in percent phosphorylase a (approximately 21% and 13% phosphorylase a for diabetic and normal myocytes, respectively). When normal and alloxan-diabetic myocytes were maintained in primary culture for 24 hours in the absence or presence of 10 μM palmitoylcarnitine or 1 mM carnitine, the phosphorylase activation response to epinephrine stimulation was largely unaffected. As shown in **Figure 11 A** for normal and **Figure 11 B** for alloxan-diabetic cardiomyocytes, the addition of palmitoylcarnitine or carnitine to myocyte cultures had a statistically insignificant effect on decreasing the magnitude of the phosphorylase response to epinephrine challenge. After 24 hours in culture, the phosphorylase response to epinephrine stimulation in both normal and alloxan-diabetic myocytes, with or without palmitoylcarnitine or carnitine, was approximately 30% phosphorylase a.

Previous reports have demonstrated that the administration of insulin to chronically diabetic rats could prevent or reverse altered myocardial function in the diabetic heart (86). The next series of experiments was undertaken to determine if fatty acid metabolism in diabetic myocytes could be stimulated upon the addition of insulin. For these studies, normal and alloxan-diabetic myocytes were cultured for 1 additional hour in the absence or presence of 1 mM carnitine, 100 nM insulin or with carnitine and insulin. As shown in **Figure 12 A** for normal and **Figure 12 B** for alloxan-diabetic cardiomyocytes, the addition of carnitine, insulin or the combination of these two agents, was not effective in altering the response of phosphorylase to epinephrine stimulation. A similar trend was

observed for normal (**Figure 13 A**) and diabetic myocytes (**Figure 13 B**) after maintaining the cells for 24 hours in the absence or presence of carnitine, insulin or carnitine with insulin prior to challenge with epinephrine.

Lastly, to determine if carnitine palmitoyltransferase 1 (CPT-1) activity was affected in these cells, normal and alloxan-diabetic cardiomyocytes were maintained in culture in the absence or presence of etomoxir. Etomoxir irreversibly inhibits palmitoylcarnitine transferase activity with the net result being the accumulation of long chain acyl CoA's within the cell (53,56). The actual inhibitor is not etomoxir but the corresponding CoA ester which is formed *in vivo* (51). Preincubation of normal (**Figure 14 A**) and alloxan-diabetic cardiomyocytes (**Figure 14 B**) without or with etomoxir concentrations ranging from 1 nM to 10 μM, did not affect the phosphorylase activation response to epinephrine after the cells were maintained in culture for 3 hours. Maintenance of normal (**Figure 15 A**) and alloxan-diabetic cardiomyocytes (**Figure 15 B**) in the absence or presence of etomoxir for 24 hours also failed to alter epinephrine-stimulated phosphorylase activation. Collectively, these data fail to support a crucial role for fatty acid metabolite involvement in the activation of glycogen phosphorylase in normal and acute, alloxan-diabetic cultured cardiomyocytes.

C. Discussion.

The present study demonstrates that the presence of endogenous fatty acid metabolites does not affect phosphorylase activation in normal- and acute, alloxan-diabetic-derived cardiomyocyte cultures. When carnitine or palmitoylcarnitine was added to 3 and 24 hour normal and alloxan-diabetic

cardiomyocytes, the subsequent activation of phosphorylase by epinephrine was largely unaffected. The addition of exogenous carnitine in both the absence and presence of insulin was also unsuccessful in attenuating the phosphorylase response. Similarly, inhibition of carnitine palmitoyltransferase 1 activity by etomoxir was shown not to affect phosphorylase activation in normal and diabetic myocyte cultures. While the activity of several key enzymes including Ca^{+2}-Mg^{+2}-ATPase (52) and signal transduction via arachidonic acid and leukotriene cascades (49) are affected by the accumulation of long chain acylcarnitines and acyl CoA's in the diabetic heart, these studies indicate that glycogen phosphorylase activity is not affected by the presence of these compounds in cardiomyocytes isolated from normal and acute (5 day), alloxan-diabetic animals. To what extent phosphorylase activity is affected by the presence of these agents in chronic diabetic animals is unknown.

Earlier studies have indicated that chronic administration of L-carnitine to diabetic rats had a protective effect against the myocardial damage which is associated with the onset of diabetes (75,76). While the precise mechanism for the improvement in cardiac function in these animals is not known, the protective effect was shown to be dependent on the duration of carnitine treatment and the concentration of carnitine administered (75). While the present studies investigated the effects of high dose carnitine treatment on phosphorylase activation, maintenance of myocytes in culture for periods up to 48 hours in the presence of carnitine was shown not to affect phosphorylase activation (data not shown). While carnitine supplementation may exert beneficial effects in the hearts from chronically diabetic rats, a major role for carnitine involvement in

phosphorylase activation in primary culture, acute, diabetic cardiomyocytes seems unlikely.

The lack of an effect by insulin with or without carnitine on phosphorylase activation was not suprising. It had been shown that when cultures of normal and alloxan-diabetic cardiomyocytes were supplemented with insulin prior to epinephrine stimulation, the subsequent activation of phosphorylase was unaffected (11). Although other studies did suggest that insulin treatment could prevent some of the more adverse cardiac effects which are associated with chronic diabetes, insulin therapy was shown to be effective only when insulin treatment was initiated with the onset of disease (86). Since both insulin and carnitine were added to ventricular myocyte cultures several hours after the cells were placed into primary culture, it seems quite likely that these agents elicit their protective effects on the heart *in vivo* but not *in vitro*.

In isolated working hearts, inhibition of carnitine palmitoyltransferase 1 activity by etomoxir was shown to significantly improve myocardial function in acute and chronic diabetic, ischemic hearts (54,56). This effect was shown to be concentration dependent (54,56). When lower (nanomolar) concentrations of etomoxir were infused into the diabetic hearts, there was a marked decrease in long chain acylcarnitine levels with little or no effect on cardiac function (53,54,56). At higher (micromolar) concentrations of etomoxir, myocardial function improved significantly, however, improved function was not correlated with a decrease in long chain acylcarnitines and acyl CoA's (53,54,56). The lack of reduction of the acyl compounds at high etomoxir concentrations in all probability resulted from the inhibition of not only CPT-1 but CPT-2 as well (**Figure 9**) (54,56). In the present study, the effects of high and low etomoxir

concentrations on phosphorylase activation in normal and alloxan-diabetic myocyte cultures was assessed. The data suggest that etomoxir does not alter phosphorylase activation under these conditions. Collectively, these studies demonstrate that the presence of fatty acid metabolites does not affect phosphorylase activation by epinephrine in normal and acute alloxan-diabetic-derived ventricular myocyte cultures.

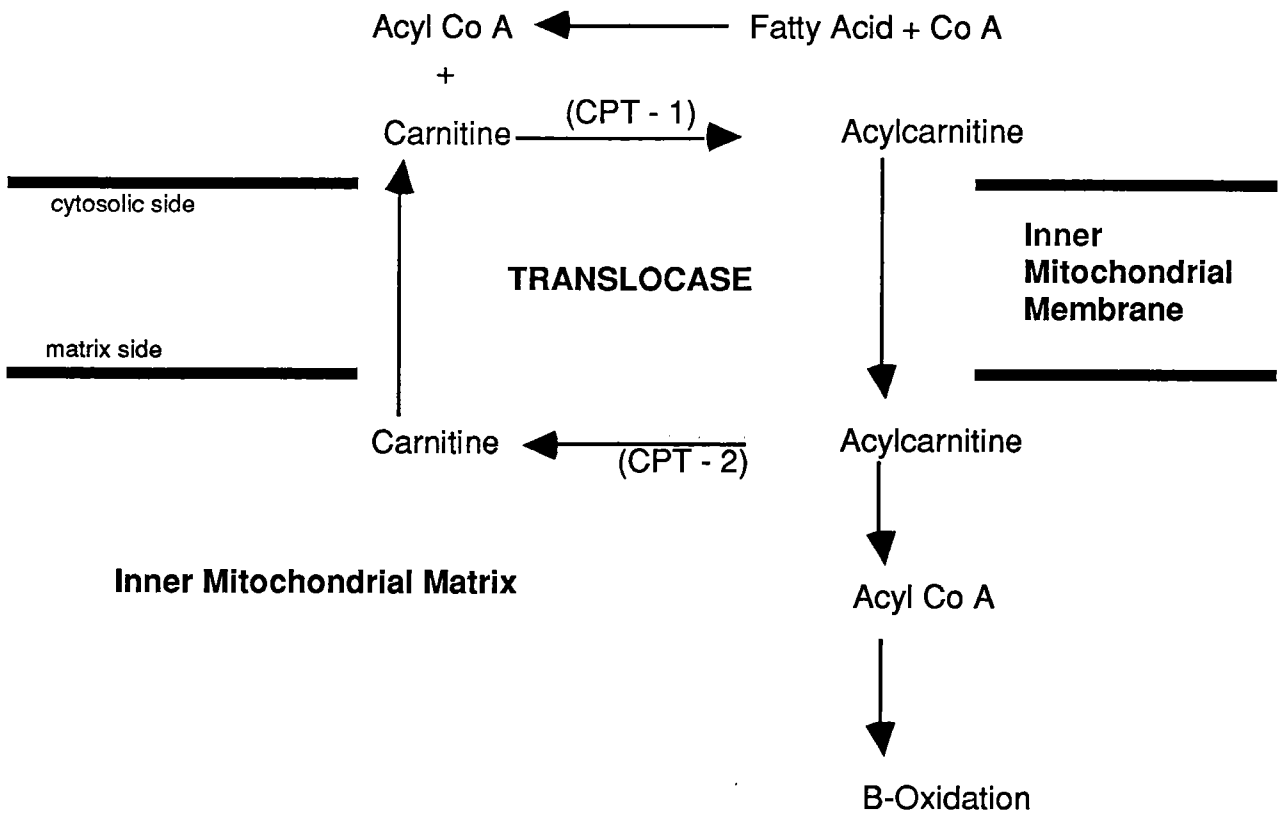

Figure 9. **Overview of fatty acid metabolism.**

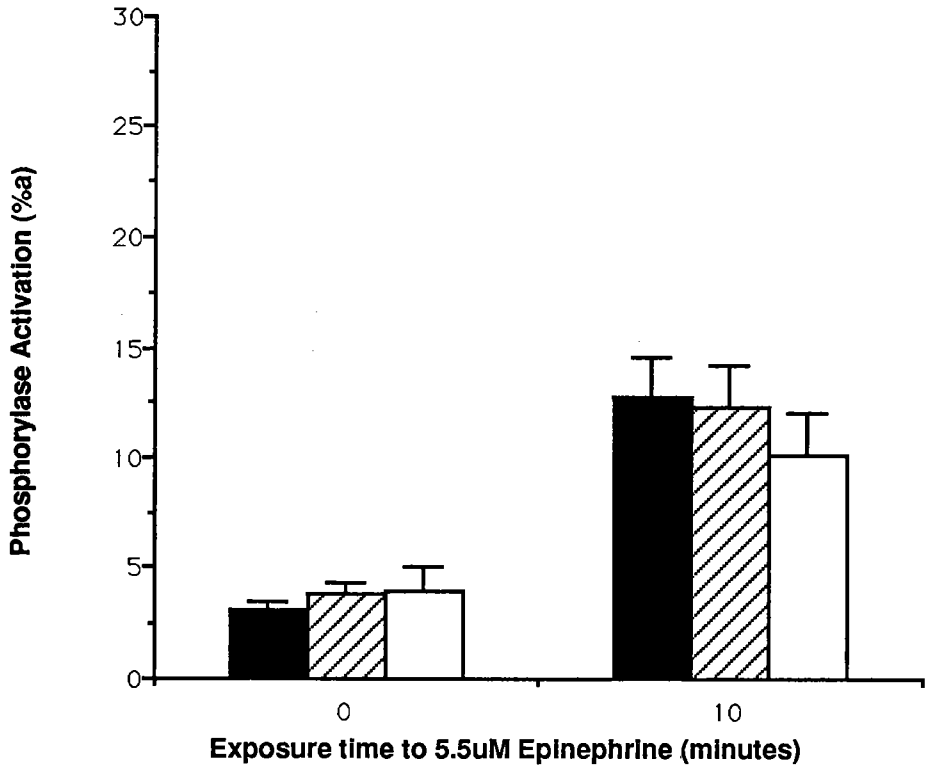

Figure 10 A. **Effect of palmitoylcarnitine and carnitine on epinephrine-stimulated phosphorylase activation in normal cardiomyocytes after 3 hours in culture.** Cardiomyocytes from normal animals were isolated and maintained in culture for 2 hours prior to equilibration in supplemented MEM without (closed bars) or with 10 μM palmitoylcarnitine (hatched bars) or 1 mM carnitine (open bars) for 1 hour. Three hour cells were then challenged with 5.5 μM epinephrine for 10 minutes and assayed for phosphorylase activity. Plotted values represent the mean ± S.E.M. where n = 6.

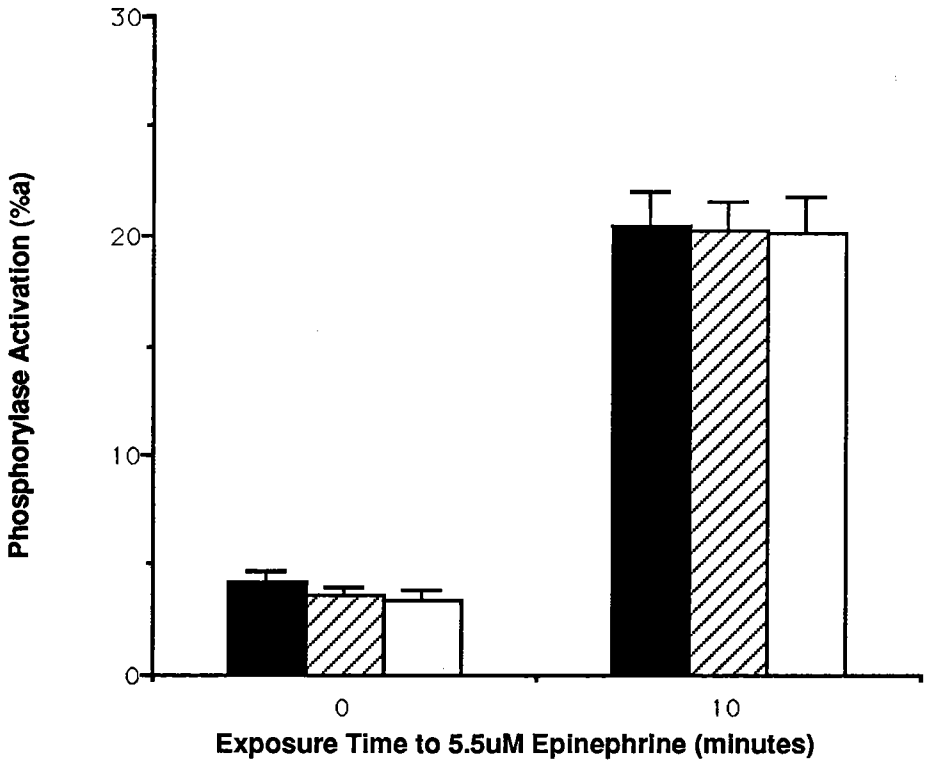

Figure 10 B. **Effect of palmitoylcarnitine and carnitine on epinephrine-stimulated phosphorylase activation in alloxan-diabetic cardiomyocytes after 3 hours in culture.** Cardiomyocytes from diabetic animals were isolated and maintained in culture for 2 hours prior to equilibration in supplemented MEM without (closed bars) or with 10 μM palmitoylcarnitine (hatched bars) or 1 mM carnitine (open bars) for 1 hour. Three hour cells were then challenged with 5.5 μM epinephrine for 10 minutes and assayed for phosphorylase activity. Plotted values represent the mean ± S.E.M. where n = 6.

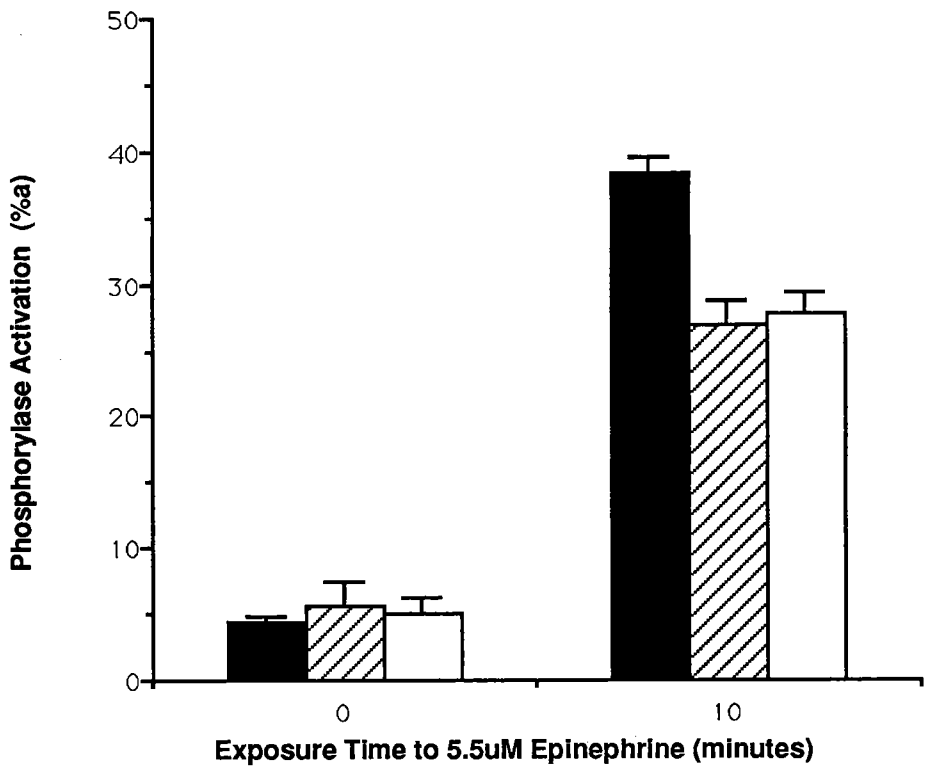

Figure 11 A. **Effect of palmitoylcarnitine and carnitine on epinephrine-stimulated phosphorylase activation in normal cardiomyocytes after 24 hours in culture.** Cardiomyocytes from normal animals were isolated and maintained in culture for 24 hours in the absence (closed bars) or presence of either 10 μM palmitoylcarnitine (hatched bars) or 1 mM carnitine (open bars). Cells were challenged with 5.5 μM epinephrine for 10 minutes and were assayed for phosphorylase activity. Plotted values represent the mean ± S.E.M. where n = 6.

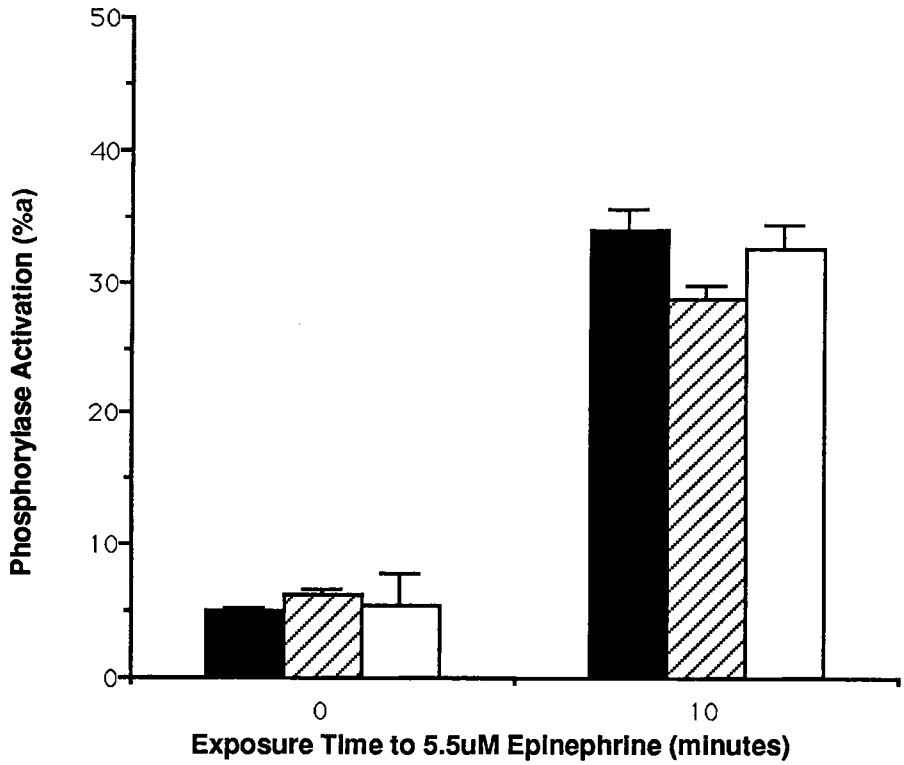

Figure 11 B. **Effect of palmitoylcarnitine and carnitine on epinephrine-stimulated phosphorylase activation in alloxan-diabetic cardiomyocytes after 24 hours in culture.** Cardiomyocytes from diabetic animals were isolated and maintained in culture for 24 hours in the absence (closed bars) or presence of either 10 μM palmitoylcarnitine (hatched bars) or 1 mM carnitine (open bars). Cells were challenged with 5.5 μM epinephrine for 10 minutes and were assayed for phosphorylase activity. Plotted values represent the mean ± S.E.M. where n = 6.

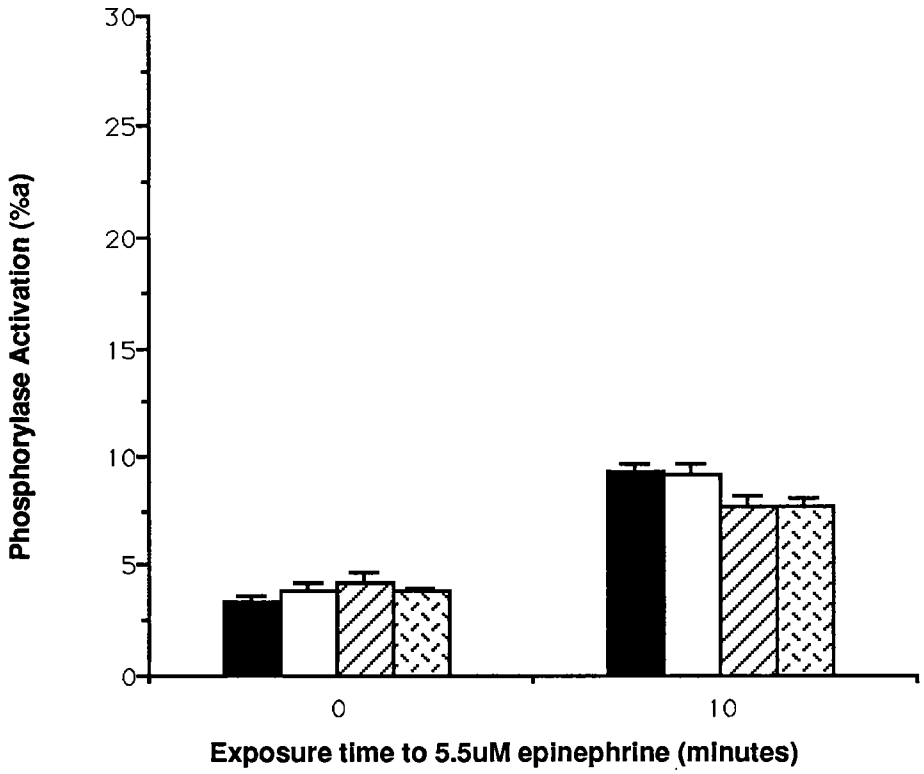

Figure 12 A. **Effect of carnitine and insulin on epinephrine-stimulated phosphorylase activation in normal cardiomyocytes after 3 hours in culture.** Myocytes from normal animals were isolated and maintained in primary culture for 2 hours prior to equilibration with media without (closed bars) or with 1 mM carnitine (open bars), 100 nM insulin (hatched bars) or both carnitine and insulin (speckled bars). Three hour myocytes were challenged for 10 minutes with 5.5 μM epinephrine and assayed for phosphorylase activity. Plotted values represent the mean ± S.E.M. where n = 6.

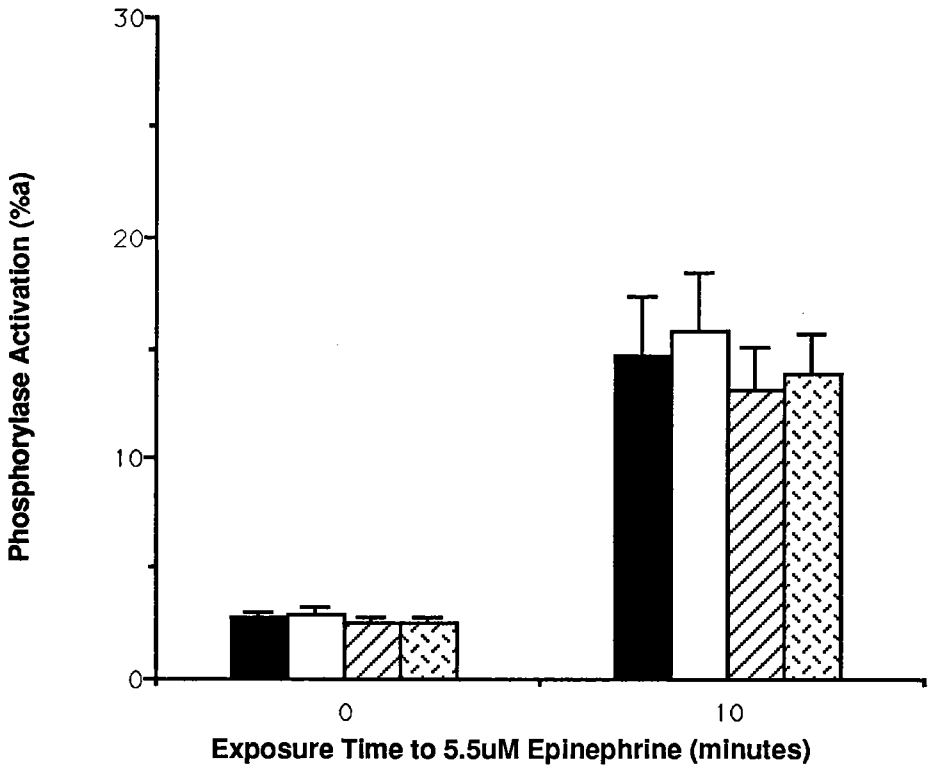

Figure 12 B. **Effect of carnitine and insulin on epinephrine-stimulated phosphorylase activation alloxan-diabetic cardiomyocytes after 3 hours in culture.** Myocytes from diabetic animals were isolated and maintained in primary culture for 2 hours prior to equilibration with media without (closed bars) or with 1 mM carnitine (open bars), 100 nM insulin (hatched bars) or both carnitine and insulin (speckled bars). Three hour myocytes were challenged for 10 minutes with 5.5 μM epinephrine and assayed for phosphorylase activity. Plotted values represent the mean ± S.E.M. where n = 6.

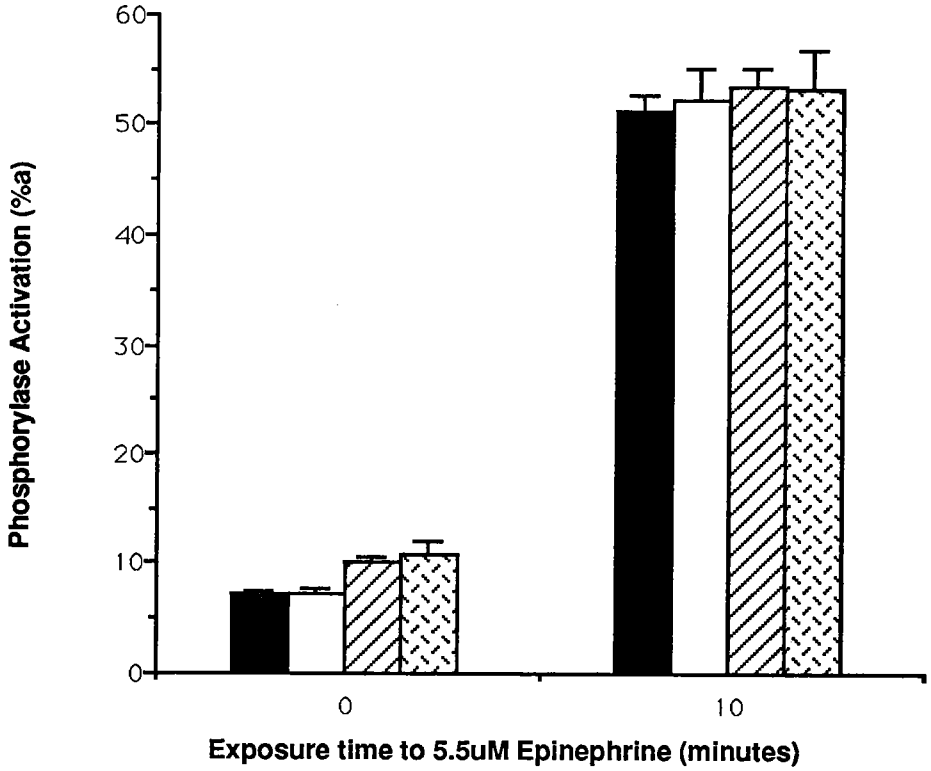

Figure 13 A. **Effect of carnitine and insulin on epinephrine-stimulated phosphorylase activation in normal cardiomyocytes after 24 hours in culture.** Cardiomyocytes from normal animals were maintained in culture for 24 hours in the absence (closed bars) or presence of 1 mM carnitine (open bars), 100 nM insulin (hatched bars) or both carnitine and insulin (speckled bars). Cells were challenged with 5.5 μM epinephrine for 10 minutes and assayed for phosphorylase activity. Plotted values represent the mean ± S.E.M. where n = 6.

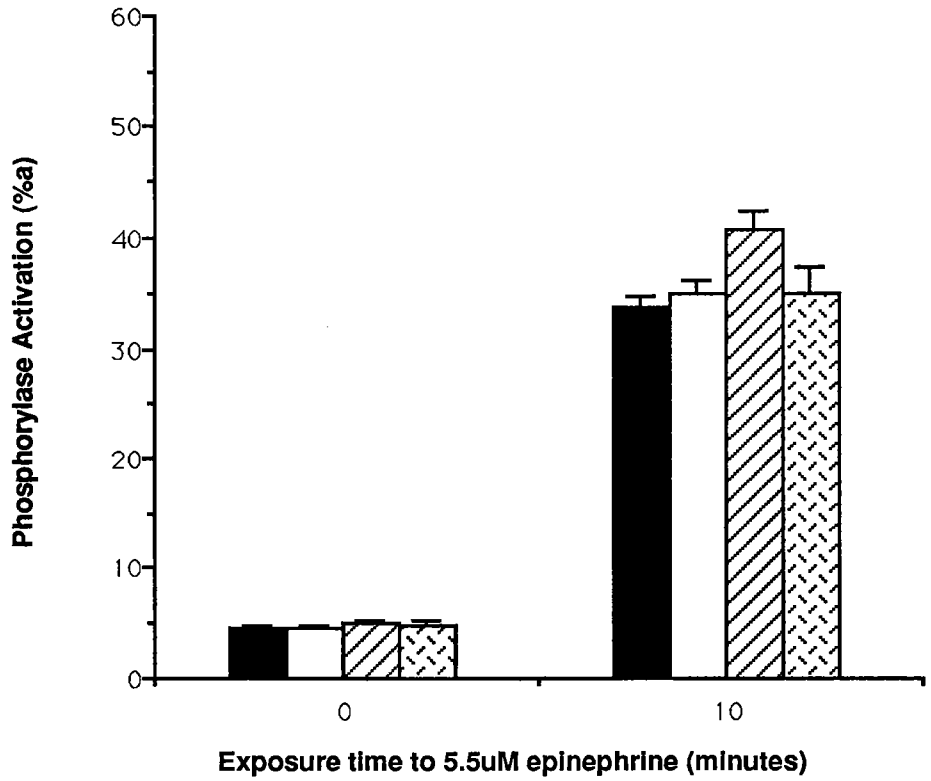

Figure 13 B. **Effect of carnitine and insulin on epinephrine-stimulated phosphorylase activation in alloxan-diabetic cardiomyocytes after 24 hours in culture.** Cardiomyocytes from diabetic animals were maintained in culture for 24 hours in the absence (closed bars) or presence of 1 mM carnitine (open bars), 100 nM insulin (hatched bars) or both carnitine and insulin (speckled bars). Cells were challenged with 5.5 μM epinephrine for 10 minutes and assayed for phosphorylase activity. Plotted values represent the mean ± S.E.M. where n = 6.

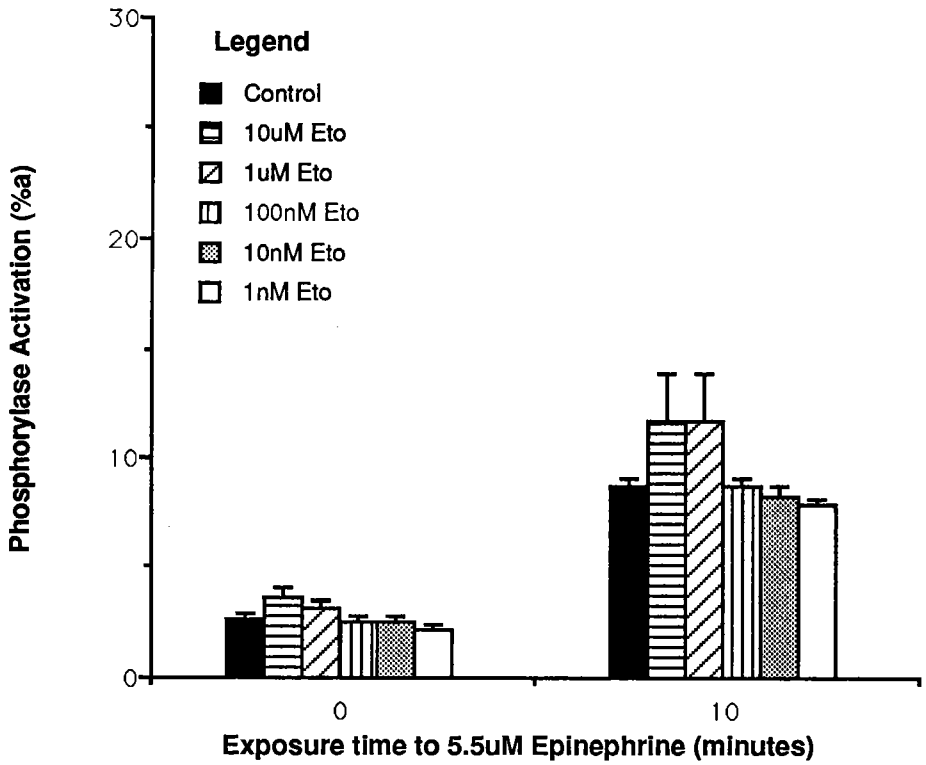

Figure 14 A. **Effect of etomoxir on epinephrine-stimulated phosphorylase activation in normal cardiomyocytes after 3 hours in culture.** Cardiomyocytes from normal animals were maintained in primary culture for 2 hours prior to the addition of etomoxir. Cells were equilibrated for 1 additional hour prior to challenge with 5.5 μM epinephrine for 10 minutes. The myocytes were assayed for phosphorylase activity. Plotted values represent the mean ± S.E.M. where n = 6.

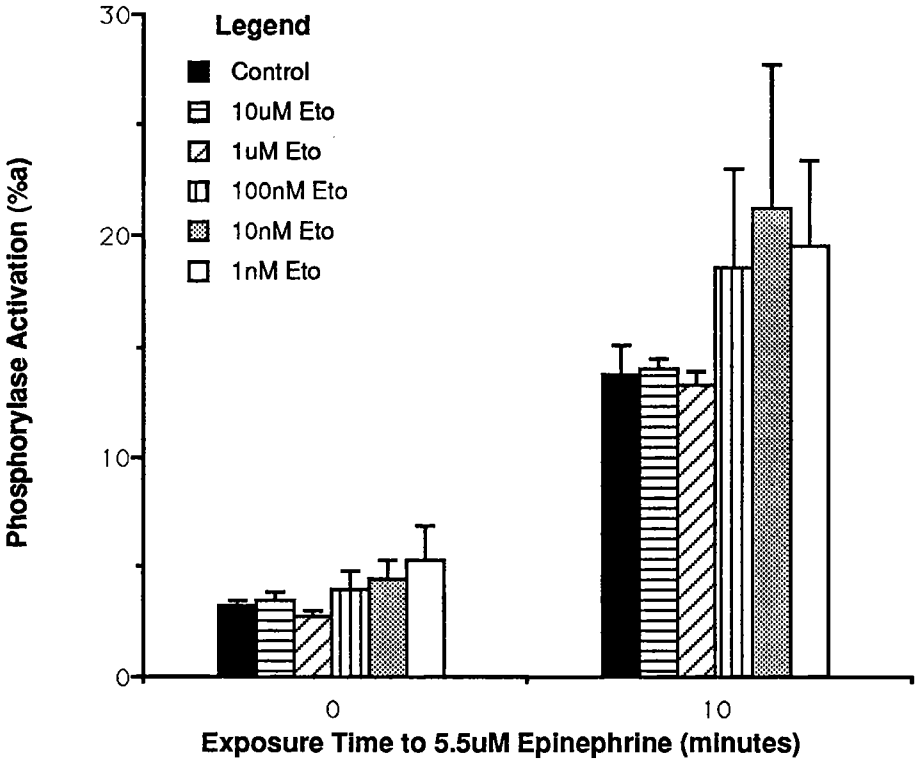

Figure 14 B. **Effect of etomoxir on epinephrine-stimulated phosphorylase activation in alloxan-diabetic cardiomyocytes after 3 hours in culture.** Cardiomyocytes from diabetic animals were maintained in primary culture for 2 hours prior to the addition of etomoxir. Cells were equilibrated for 1 additional hour prior to challenge with 5.5 μM epinephrine for 10 minutes. The myocytes were assayed for phosphorylase activity. Plotted values represent the mean ± S.E.M. where n = 6.

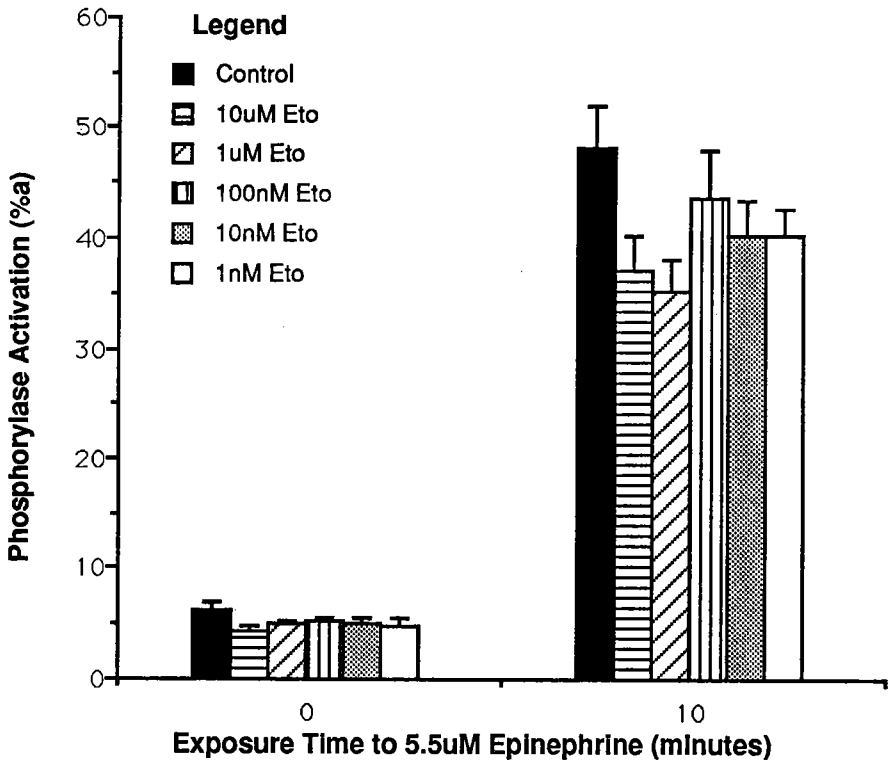

Figure 15 A. **Effect of etomoxir on epinephrine-stimulated phosphorylase activation in normal cardiomyocytes after 24 hours in culture.** Cells from normal animals were maintained in culture for 24 hours in the absence (control) or presence of etomoxir. Myocytes were challenged with 5.5 μM epinephrine for 10 minutes and the cells were assayed for phosphorylase activity. Plotted values represent the mean ± S.E.M. where n = 6.

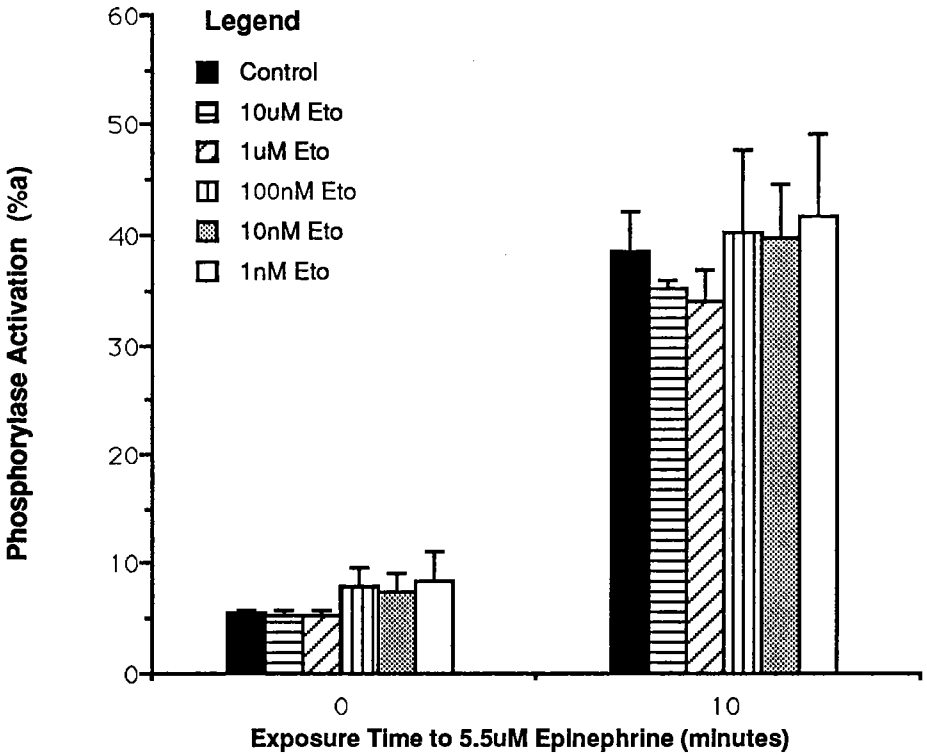

Figure 15 B. **Effect of etomoxir on epinephrine-stimulated phosphorylase activation in alloxan-diabetic cardiomyocytes after 24 hours in culture.** Cells from diabetic animals were maintained in culture for 24 hours in the absence (control) or presence of etomoxir. Myocytes were challenged with 5.5 μM epinephrine for 10 minutes and the cells were assayed for phosphorylase activity. Plotted values represent the mean ± S.E.M. where n = 6.

CHAPTER V

Potential for G-Protein Involvement in Phosphorylase Activation: Evidence for a Post-Receptor Defect

A. Introduction.

Glycogen metabolism is acutely regulated by several hormones which promote the synthesis of glycogen for storage or its degradation for use as a cellular energy source (20,27). Glycogen phosphorylase, the enzyme directly responsible for glycogen breakdown, is regulated in the heart by epinephrine, a circulating catecholamine, which activates the enzyme *via* the β-adrenergic receptor system (7,11,17,68). While the actual number of surface β-receptors is significantly decreased in the diabetic myocardium (48,66,78), it has been shown that glycogen phosphorylase activity in isolated, alloxan-diabetic cardiomyocytes, as well as in other diabetic heart preparations, responds to epinephrine stimulation in a hypersensitive fashion (5,11,63,64). Earlier studies carried out in this laboratory have shown that cells derived from alloxan-diabetic animals exhibit a hypersensitive glycogen phosphorylase response to epinephrine challenge which was apparent 3 hours after cell isolation and was further enhanced upon maintenance of these cells in primary culture for 24 hours (11,89). Cells derived from normal animals initially lacked this response to epinephrine after 3 hours in culture although maintenance of these cells in culture for 24 hours resulted in an *in vitro* acquisition of the hypersensitive response (11).

While the response to catecholamine stimulation has been shown to be mediated through the activation of surface β-adrenergic receptors (11), the physiological basis for the hypersensitive response is unknown.

Activation of glycogen phosphorylase by epinephrine requires the coupling of surface β-receptors to the adenylyl cyclase cascade system by receptor-linked guanine nucleotide regulatory or G-proteins (4,15). For hormones such as epinephrine which utilize cAMP as an intracellular messenger, two groups of G-proteins, Gs and Gi, are known to mediate the receptor-dependent stimulation and inhibition of adenylyl cyclase activity, respectively (34,81). ADP-ribosylation of Gs by cholera toxin or Gi by pertussis toxin, directly affects the ability of these proteins to couple efficiently to their receptor and effector systems (4,15). Any alteration in the coupling between the appropriate G-protein and adenylyl cyclase, which is responsible for intracellular cAMP production, could ultimately influence the subsequent activation of glycogen phosphorylase.

The aim of this study was to determine if the hypersensitive response of glycogen phosphorylase activation to catecholamine stimulation could result from altered signal transduction through the β-adrenergic receptor system or from a post-receptor defect. The present work demonstrates that while Gs mediates the hypersensitive activation of glycogen phosphorylase in response to epinephrine stimulation, the hypersensitive response which is observed in alloxan-diabetic cardiomyocytes and is induced *in vitro* in normal myocytes is due to a defect at a post-receptor site.

B. Results.

To assess the potential for G-protein involvement in the hypersensitive response of glycogen phosphorylase to catecholamine stimulation, the data presented in **Figure 16** illustrate the effect of cholera toxin on the epinephrine-induced activation of glycogen phosphorylase for normal and alloxan-diabetic cardiomyocytes maintained in primary culture for 3 hours. In cells not exposed to cholera toxin, the alloxan-diabetic cells exhibited almost a two-fold greater response than the normal cells to epinephrine as reflected by the increase in phosphorylase a (17.5% vs. 10.5% for alloxan-diabetic and normal myocytes, respectively). When normal and alloxan-diabetic cells were incubated with 25 μg/ml cholera toxin for 1 hour prior to epinephrine stimulation, both normal and alloxan-diabetic cells exhibited a potentiated response to the hormone (30.4% vs. 18.3% phosphorylase a for alloxan-diabetic and normal cells respectively). It is interesting to note that the response of phosphorylase was enhanced to the same extent in both normal and alloxan-diabetic myocytes after 1 hour pretreatment with cholera toxin. The phosphorylase a response for both normal and alloxan-diabetic, cholera toxin treated myocytes was 174% of non-toxin treated control cells. Incubation of both normal and alloxan-diabetic cardiomyocytes with cholera toxin for periods up to 1 hour in the absence of epinephrine stimulation failed to elicit a change in phosphorylase a (data not shown). Maintenance of normal cardiomyocytes in the presence of 25 μg/ml cholera toxin for 24 hours failed to alter the *in vitro* induction of the hypersensitive response of phosphorylase to epinephrine whereas diabetic derived

cardiomyocytes were not viable after incubation with cholera toxin for 24 hours (data not shown).

To determine if an inhibitory G-protein, namely Gi, could be involved in the activation of phosphorylase, both normal and alloxan-diabetic cardiomyocytes were incubated with 1 μg/ml pertussis toxin for 1 hour prior to stimulation with epinephrine. As shown in **Figure 17**, preincubation of the cells with pertussis toxin did not affect the myocyte response to epinephrine challenge suggesting that the inhibitory G-protein, Gi, was not involved in the activation of phosphorylase. Collectively, these data implicate a direct role for a cholera toxin sensitive G-protein, probably Gs, in the activation of glycogen phosphorylase in response to catecholamine challenge in both normal and alloxan-diabetic cardiomyocytes.

To determine if the hypersensitive response of glycogen phosphorylase to hormonal stimulation resulted from altered signal transduction through the β-adrenergic receptor system or from a defect present at a post-receptor site, normal and alloxan-diabetic cardiomyocytes were challenged with forskolin, a cardioactive diterpene which directly stimulates the adenylyl cyclase catalytic subunit activity independently of Gs (80). **Figure 18** illustrates the dose response relationship for forskolin-stimulated activation of glycogen phosphorylase for both normal and alloxan-diabetic cardiomyocytes maintained in primary culture for 3 hours. Both normal and alloxan-diabetic myocytes were sensitive to the effects of forskolin over the concentration range of 1×10^{-7} M to 1×10^{-4} M (1×10^{-4} M forskolin was the highest concentration assayed due to solubility constraints). The response to forskolin in these cells was rapid and maximal within 5 minutes of challenge (data not shown). For normal myocytes, after three hours in culture,

the EC_{50} for forskolin-stimulated phosphorylase activation was estimated to be 4.7×10^{-5} M forskolin whereas, alloxan-diabetic myocytes maintained in culture for 3 hours were more sensitive to forskolin activation since the half maximal response was elicited with 2.2×10^{-6} M forskolin. The maximal phosphorylase a response for forskolin-stimulated enzyme activation for 3 hour, normal and alloxan diabetic cardiomyocytes was 2-3 fold greater than enzymatic activation in response to epinephrine challenge (11).

When cardiomyocytes were maintained in culture for 24 hours, the phosphorylase response for normal and alloxan-diabetic myocytes to forskolin was identical and is shown in **Figure 19**. The half maximal response for phosphorylase activation was elicited with 2.6×10^{-6} M and 1.3×10^{-6} M forskolin for normal and diabetic myocytes, respectively, which does not represent a statistically significant difference. These data collectively suggest that the hypersensitive response of glycogen phosphorylase to epinephrine challenge results from a defect at a post-receptor site occurring after the activation of adenylyl cyclase.

C. Discussion.

The present study implicates a direct role for Gs mediation of the hypersensitive response of glycogen phosphorylase to epinephrine stimulation in alloxan-diabetic cardiomyocytes as well as in normal myocytes. Pretreatment of cardiomyocytes with pertussis toxin prior to epinephrine challenge failed to affect phosphorylase activation whereas pretreatment with cholera toxin resulted in a potentiated response. This data suggested that a cholera toxin-sensitive G-

protein, probably Gs, mediates the hypersensitive response of glycogen phosphorylase to catecholamine stimulation in diabetic-derived cardiomyocytes. The absence of an effect with pertussis toxin was not suprising since the hypersensitive response of phosphorylase to catecholamine stimulation is mediated by the activation of surface β-adrenergic receptors (11). These results are also consistent with the findings of Hazeki and Ui (40) in that cAMP accumulation was shown not to be significantly different from control in short term cardiomyocyte cultures treated with pertussis toxin. Since the response of phosphorylase was potentiated by the same magnitude in both normal and alloxan-diabetic cardiomyocytes treated with cholera toxin, it seems unlikely that the primary defect in the hypersensitive response of glycogen phosphorylase in the diabetic heart results from altered Gs function. While Gs may mediate the activation of glycogen phosphorylase in response to epinephrine, the hypersensitive response which is present in the alloxan-diabetic cells and is induced *in vitro* in normal cardiomyocytes appears to result principally from a defect at a post-receptor site which lies after the activation of adenylyl cyclase in this signaling pathway.

When normal and alloxan-diabetic cardiomyocytes were challenged with forskolin, which directly stimulates adenylyl cyclase catalytic activity (80), alloxan-diabetic cells, cultured for 3 hours, exhibited a hypersensitive phosphorylase activation response. Normal cardiomyocytes, at 3 hours, were initially less sensitive to forskolin challenge than the alloxan-diabetic cells, however, maintenance of these cells in culture for 24 hours resulted in an *in vitro* induction of the phosphorylase response to a similar magnitude as the diabetic myocytes. When alloxan-diabetic cardiomyocytes were maintained in primary culture for 3

hours, the hypersensitive response to forskolin was due only to a decrease in EC_{50} relative to the controls whereas, when the cells were stimulated with epinephrine, the enhanced sensitivity of phosphorylase was manifested by both a decrease in EC_{50} and a two-fold increase in the maximal phosphorylase a response (11). In addition, at 24 hours, there was a smaller leftward shift in the EC_{50} for forskolin-stimulated phosphorylase activation which was accompanied by only a modest increase in maximal phosphorylase a response. While the difference between epinephrine- and forskolin-induced activation of phosphorylase may, in part, be attributed to inherent differences in their sites of action (receptor-dependent vs. receptor-independent), published reports have suggested that differences exist between the ability of forskolin and β-adrenergic amines to increase cAMP and activate cAMP-dependent protein kinase in the perfused rat heart (26). While the compartmentalization of cAMP in the heart could account for the differences observed for the hypersensitive response of phosphorylase to epinephrine and forskolin stimulation, further studies are required to resolve this complex issue.

Since the hypersensitivity of glycogen phosphorylase in the diabetic heart does not appear to result from a direct increase in surface β-receptors (48,66,78,87), modified G-protein mRNA levels (37,70), or altered signal transduction *via* the β-adrenergic receptor (44), one alternate mechanism which could account for the hypersensitive response of phosphorylase is through the alteration of cytosolic free calcium levels. Recent studies have shown that the intracellular calcium concentration is greater in cardiomyocytes derived from diabetic animals when compared to control animals based on fura-2 fluorescence measurements (1). In addition, abnormal cardiac calcium-metabolism in the

diabetic state has been well documented with defects present at several key regulatory points (43,59). Changes in calcium channel flux (67,88), and sarcoplasmic reticular calcium transport in the diabetic state (31,43,52,55), could each individually, or in concert, serve to increase intracellular calcium to a point at which calcium-dependent and calcium-sensitive systems, such as phosphorylase kinase, would respond in a hypersensitive fashion. Conversely, if altered calcium homeostasis is to be directly implicated in the hypersensitive response, one might expect to see an altered phosphorylase activation response following epinephrine challenge after diabetic heart preparations are exposed to sub physiological extracellular calcium concentrations. Previous studies have indicated that when hearts from alloxan-diabetic animals were perfused with one-third of the normal perfusate calcium concentration, the hypersensitivity of phosphorylase activation to epinephrine was partially reversed (63).

While the hypersensitivity of the glycogen phosphorylase activation pathway in response to direct catecholamine challenge appears to be an intrinsic property of the diabetic heart, and not an isolation artifact, the possibility exists that calcium homeostasis may be altered upon maintenance of these cells in primary culture. Intracellular free calcium levels may be initially higher in cells derived from alloxan-diabetic animals which would explain the hypersensitive phosphorylase response to epinephrine which is exhibited by these cells 3 hours after isolation as well as in other diabetic heart preparations (61,63,64,81). Maintenance of alloxan-diabetic cells, as well as normal cardiomyocytes, in primary culture for 24 hours could result in the alteration of calcium channel function or the phosphorylation states of one or more of the proteins which are directly involved in maintaining intracellular free calcium concentrations.

Increased intracellular calcium concentrations could also serve to promote a significant change in phosphorylase kinase activity which is subject to regulation, in part, by calcium (41). Similarly, changes in the activities or localization of the enzymes responsible for maintaining the phosphorylation state of phosphorylase kinase, could promote the hypersensitive activation of phosphorylase. While cAMP and cAMP-dependent protein kinase activity is not significantly affected in the diabetic state based on studies in perfused hearts (45,63) and in isolated myocytes (79), changes in phosphorylase kinase phosphatase activity, the enzyme responsible for dephosphorylating and inactivating phosphorylase kinase, could be reflected as a change in activity at the level of phosphorylase. Differences in the intracellular localization of phosphorylase or phosphorylase kinase could also affect the kinetics and/or magnitude of the observed response to epinephrine.

While the precise physiological basis for the hypersensitivity phenomenon is unknown, an alteration in one or several of the cellular components which regulate intracellular calcium levels and/or phosphorylase kinase activity may promote the hypersensitive activation of glycogen phosphorylase in the diabetic heart. Further investigation is warranted to determine the precise role of phosphorylase kinase and calcium in this pathway and whether alterations in their regulation are responsible for the diabetes-associated defect in phosphorylase activation.

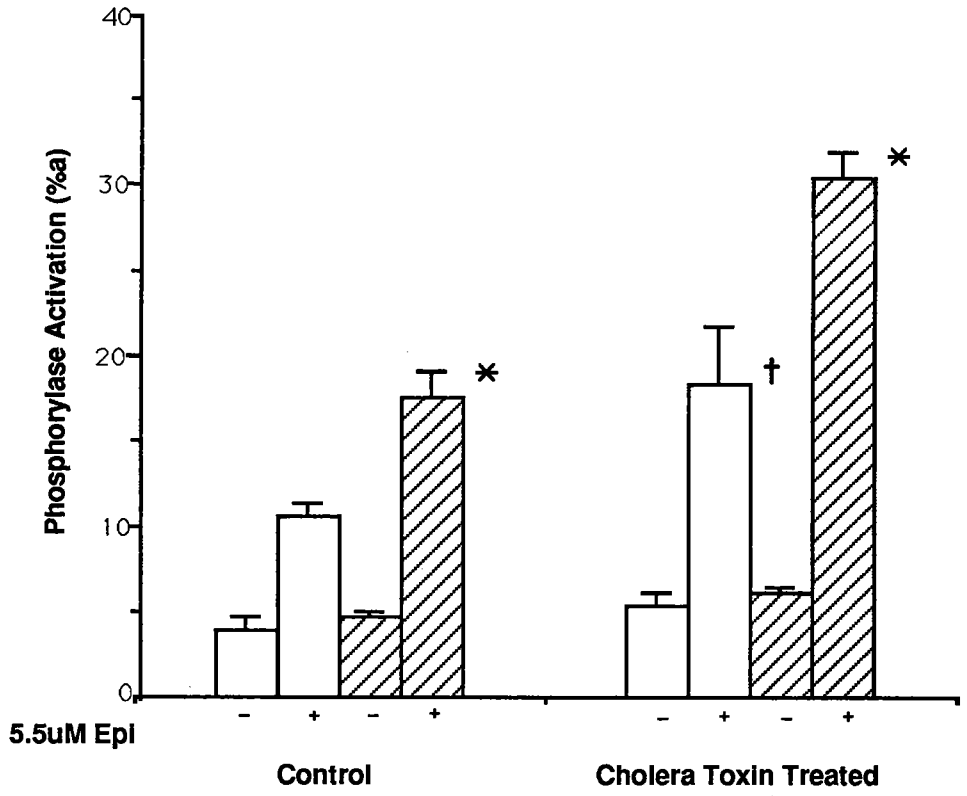

Figure 16. **Effect of cholera toxin on epinephrine-stimulated phosphorylase activation in normal and alloxan-diabetic cardiomyocytes after 3 hours in culture.** Cardiomyocytes from normal (open bars) and alloxan-diabetic animals (hatched bars) were maintained in culture for 2 hours prior to equilibration with supplemented MEM with 0.2 mM palmitate, 0.24% albumin with or without 25 µg/ml cholera toxin, for 1 hour. Cardiomyocytes, cultured for 3 hours, were then challenged with 5.5 µM epinephrine **(+ Epi)** for 10 minutes and assayed for phosphorylase activity. Plotted values represent the mean ± S.E.M. where n = 6 for normal and n = 8 for alloxan-diabetic cell preparations. * $P < 0.005$ *vs.* normal + epinephrine. † $P < 0.025$ *vs.* normal + epinephrine.

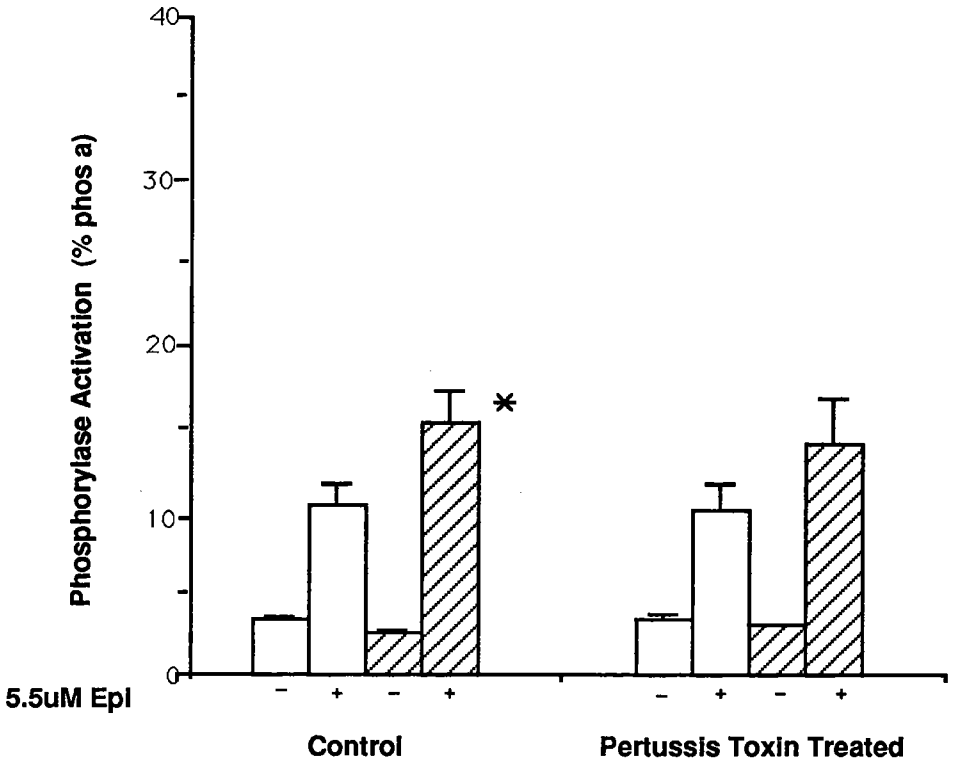

Figure 17. **Effect of pertussis toxin on epinephrine-stimulated phosphorylase activation in normal and alloxan-diabetic cardiomyocytes after 3 hours in culture.** Cardiomyocytes from normal (open bars) and alloxan-diabetic animals (hatched bars) were isolated and maintained in culture for 2 hours prior to equilibration with supplemented MEM with 0.2 mM palmitate, 0.24% albumin with or without 1 μg/ml pertussis toxin for 1 additional hour. Cardiomyocytes, cultured for three hours, cells were then challenged with 5.5 μM epinephrine **(+Epi)** for 10 minutes and assayed for phosphorylase activity. Plotted values represent the mean ± S.E.M. where n = 6 for both normal and alloxan-diabetic cell preparations. * $P < 0.025$ *vs.* normal + epinephrine

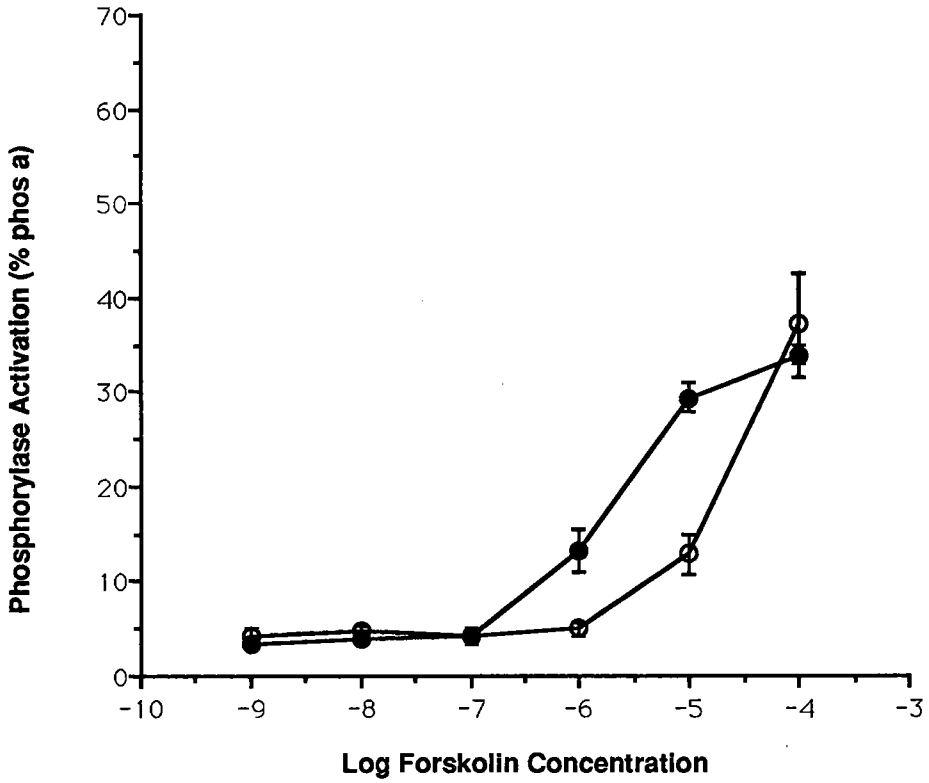

Figure 18. **Dose response relationship for forskolin-stimulated phosphorylase activation in normal and alloxan-diabetic cardiomyocytes after 3 hours in primary culture.** Cardiomyocytes from normal and alloxan-diabetic animals were prepared and equilibrated in MEM with 0.2 mM palmitate and 0.24% albumin. Normal (open circles) and alloxan-diabetic cells (closed circles) were stimulated with forskolin for 15 minutes. The myocytes were later thawed on ice and assayed for phosphorylase activity. Plotted values represent the mean $\pm$ S.E.M. where n = 6 for both normal and alloxan-diabetic cell preparations.

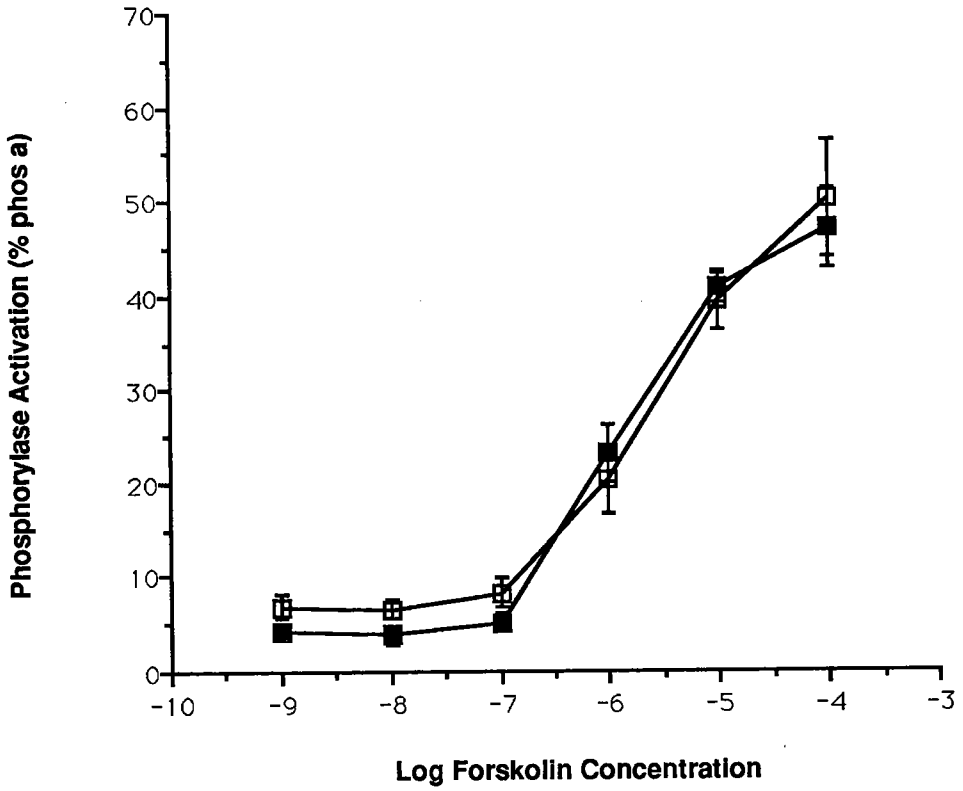

Figure 19. **Dose response relationship for forskolin-stimulated phosphorylase activation in normal and alloxan-diabetic cardiomyocytes after 24 hours in primary culture.** Cardiomyocytes were prepared from normal and alloxan-diabetic animals and were allowed to equilibrate for 24 hours in MEM with 0.2 mM palmitate and 0.24% albumin. Normal (open squares) and alloxan-diabetic cells (closed squares) were challenged with forskolin for 15 minutes. The myocytes were later thawed on ice and assayed for phosphorylase activity. Plotted values represent the mean ± S.E.M. where n = 6 for both normal and alloxan-diabetic cell preparations.

CHAPTER VI

Assessment of Cardiac Phosphorylase Kinase Activity in Response to Catecholamine Stimulation

A. Introduction.

Phosphorylase kinase plays a pivotal role in the physiological control of the glycogenolytic cascade by catalyzing the conversion of phosphorylase b to the active a form (2,58). In the heart, phosphorylase kinase exists as a complex enzyme consisting of $\alpha'_4 \cdot \beta_4 \cdot \gamma_4 \cdot \delta_4$ subunits and is regulated by both cAMP and calcium (18,84). The γ-subunit of phosphorylase kinase contains the enzyme's catalytic site while the δ-subunit, which is identical to calmodulin, is the site of calcium-dependent allosteric regulation (84). In the heart, both the α'- and β-subunits function in a regulatory capacity and are phosphorylated in response to hormonal stimuli (41,58). An increase in cardiac phosphorylase kinase activity is better correlated with the cAMP-dependent phosphorylation of both the α'- and β-subunits than with phosphorylation of either subunit alone (22,58,71).

Phosphorylase kinase activity is affected not only by the presence of allosteric effectors and covalent modification but also by pH (41). In cardiac muscle, as in skeletal muscle, phosphorylase kinase has very little activity at pH 6.8 or below and is termed "nonactivated" (**Figure 1**) (22,58). In contrast, at pH 8.2, the nonactivated form of phosphorylase kinase can undergo as much as a 50-fold increase in activity (22,58). Nonactivated phosphorylase kinase can be

converted to an activated form which has 25- to 50-fold more activity at pH 6.8 and two-fold greater activity at pH 8.2 upon phosphorylation or partial proteolysis (22,58). Phosphorylase kinase activity data typically report the ratio of the enzyme's activity at pH 6.8 to that at pH 8.2 although these values are often subject to wide dispersion of the data (84). Recent reports have indicated that the most reliable and reproducible index of phosphorylase kinase activity is to measure the specific activity of the enzyme at pH 6.8 (2,84).

Previous studies have indicated that the hypersensitive response of glycogen phosphorylase to catecholamine stimulation in the diabetic heart results from a defect present at a post-receptor site which lies after the activation of adenylyl cyclase in the cAMP second messenger pathway (10,60,64). While earlier studies had demonstrated that cAMP and cAMP-dependent protein kinase activity were unaffected by diabetes (45,64,79), this data implicated a potential role for phosphorylase kinase mediation of the hypersensitive response of phosphorylase to epinephrine challenge in the diabetic heart (64). When phosphorylase kinase activity was measured in gravity-flow perfused hearts from normal and acute alloxan-diabetic rats, phosphorylase kinase activity was shown to be two-fold greater in hearts derived from acute diabetic animals than from normal animals following perfusion with epinephrine (64).

The purpose of the present study was to measure cardiac phosphorylase kinase activity initially in perfused hearts (to optimize the assay parameters) and subsequently in isolated cardiomyocytes to assess the potential role of phosphorylase kinase in the hypersensitive activation of glycogen phosphorylase in the diabetic heart. The results demonstrate that although the present assay for phosphorylase kinase activity is an accurate and reliable method for measuring

the amount of active phosphorylase kinase activity in the heart, the assay conditions must be further optimized before this system can be applied for measurement of phosphorylase kinase activity in primary culture cardiomyocytes.

B. Results.

For these studies, measurement of phosphorylase kinase activity is based upon the determination of phosphorylase b phosphorylation *in vitro*. Therefore, the integrity of the substrate must be assessed to ensure optimal assay conditions. Partially denatured phosphorylase b would be a poorer substrate for phosphorylation by phosphorylase kinase which would be manifested as a decrease in the enzyme's specific activity (84). The data presented in **Table 2** illustrate the comparison of phosphorylase b sources for use as a substrate to assay phosphorylase kinase activity.

In these studies, hearts from normal animals were perfused with buffer with (+ Epi) or without 10 μM epinephrine (- Epi) prior to freezing in liquid nitrogen. Frozen hearts were powdered and a heart homogenate was prepared. After microcentrifugation, the heart cytosolic extracts were assayed for phosphorylase kinase activity using commercially available phosphorylase b preparations or phosphorylase b, purified to near homogeneity, which was provided by Dr. James Dobson. Phosphorylase kinase activity was routinely assayed in the absence of phosphorylase b (to determine the endogenous phosphorylase b content of the samples) and in the presence of phosphorylase

b. The data are expressed as the difference in cpm between the two experimental conditions.

As shown in **Table 2**, in the assays which used Sigma's phosphorylase b as a substrate, the buffer control, in the absence of phosphorylase b, showed minimal or background ^{32}P incorporation whereas the buffer control, in the presence of phosphorylase b showed about an 8-fold increase in the amount of incorporated label. The increase in cpm in the control samples probably represents the activity of the various contaminants (i.e. phosphorylase a, phosphorylase kinase, phosphatases etc.) which are present in this phosphorylase b preparation. When the extracts from hearts perfused without epinephrine were assayed for phosphorylase kinase activity in the absence of phosphorylase b, there was an increase in the amount of ^{32}P incorporated over that of the control, which probably reflects the presence of endogenous phosphorylase b present in the heart extract. The increase in ^{32}P incorporation could not be attributed to endogenous cAMP-dependent protein kinase activity present in the extracts since all assays contained 2 μg cAMP-dependent protein kinase inhibitor protein (PKI) in the reaction mixture (16). When the same sample was assayed, this time in the presence of phosphorylase b, ^{32}P incorporation was increased. The difference in cpm between the heart extract without epinephrine, assayed in both the absence and presence of phosphorylase b, represents the phosphorylase kinase activity which is present in the heart extract. A similar phosphorylase kinase activity trend was observed for the extract from hearts perfused with epinephrine and assayed with and without phosphorylase b. The phosphorylase kinase activity in the heart extract perfused with epinephrine was approximately 30% greater than the activity of the extract without

epinephrine. The observed increase in phosphorylase kinase activity due to epinephrine stimulation in this study is in good agreement with previous reports which measured the pH 6.8/pH 8.2 activity ratios for phosphorylase kinase in normal hearts perfused with epinephrine (64). When commercially available phosphorylase kinase was assayed under the same conditions as a positive control, significant amounts of ^{32}P incorporation into phosphorylase b was observed (data not shown).

When phosphorylase b provided by Dr. Dobson was used as a substrate for the phosphorylase kinase assay, similar results were obtained. When purified phosphorylase b was assayed in this system, the assay background was significantly lower than that obtained with the phosphorylase b from Sigma. This probably reflects the fact that this preparation was essentially free of the impurities which could influence the assay results. When phosphorylase b from Boehringer Mannheim was used as the assay substrate, enzymatic activation was not observed. The lack of enzyme activation with the Boehringer substrate probably resulted from the presence of EDTA in the lyophilized preparation which could act to inhibit phosphorylase kinase activity which is subject to regulation by calcium. What these data suggest is that the present assay for phosphorylase kinase activity is a reliable indicator of the amount of active phosphorylase kinase activity which is present in the rat heart and that the source of phosphorylase b must be carefully selected and maintained to ensure optimal assay results.

In order to determine if the hypersensitive response of phosphorylase to epinephrine stimulation was due to a defect at the level of phosphorylase kinase, cardiomyocytes from normal animals were prepared and maintained in primary culture for 3 and 24 hours with and without epinephrine stimulation. The isolated

myocytes were assayed for phosphorylase kinase activity using the phosphorylase b provided by Dr. Dobson as an assay substrate. When the myocyte suspensions were assayed for phosphorylase kinase activity, enzyme activation was not observed (data not shown). Although the amount of protein present in the assay system exceeded the limits necessary for detection, there was an apparent inhibition of phosphorylase kinase activity which was probably due to the presence of excess EDTA from the myocyte wash buffer which remained on the culture dishes when the cells were harvested from the plates. The conditions for this assay need to be further refined before this method for assaying phosphorylase kinase activity can be used in conjunction with primary culture cardiomyocytes.

C. Discussion.

The results from these studies indicate that the present method for measuring phosphorylase kinase activity is a reliable indicator of the enzyme's activity in the heart although special emphasis must be placed on maintaining the substrate's integrity. While the assay was able to measure phosphorylase kinase activity in perfused hearts, refinements are necessary before this system can be applied to primary culture cardiomyocytes.

In the present study, when phosphorylase kinase activity was measured in extracts from normal hearts perfused with epinephrine, the enzyme's activity was approximately 30% greater than the activity in extracts from hearts perfused without epinephrine. These results are consistent with other reports which describe phosphorylase kinase activity in perfused hearts in response to other

hormonal effectors including epinephrine (64), PGE_1 (60), and isoproterenol (39). While the present study did not assess phosphorylase kinase activity in the diabetic heart, previous studies have suggested that phosphorylase kinase plays a key role in the hypersensitive activation of phosphorylase to hormonal stimulation in the diabetic heart (60,63,64).

Phosphorylase kinase has long been known to be activated by the allosteric effects of calcium (24). Several studies have demonstrated that cardiac, calcium-dependent metabolism is altered in the diabetic state with defects present at multiple regulatory sites (31,43,67). Recent reports have demonstrated that the intracellular calcium concentration is greater in diabetic cardiomyocytes than in normal cardiomyocytes based on fura-2 measurements (1). Changes in sarcoplasmic reticular calcium transport (31,43), and calcium pump and channel function (43,67,88), could increase intracellular calcium levels to a point at which calcium-regulated enzymes, such as phosphorylase kinase, would respond with increased sensitivity. In turn, this enhanced sensitivity would be manifested as a change in activity at the level of phosphorylase.

The activation of phosphorylase kinase, either by an increase in calcium concentrations or by cAMP-dependent phosphorylation, results solely as a consequence of a change in the enzyme's Vmax without a change in affinity (Km) for phosphorylase (65). The kinetic properties of phosphorylase kinase are such that incremental changes in both calcium and cAMP lead to synergistic increases in the amount of active phosphorylase (65). It becomes tempting to speculate on the relevance of these observations with respect to the hypersensitive activation of phosphorylase in the diabetic heart. Upon placement of ventricular cardiomyocytes into primary culture, intracellular calcium concentrations would

be expected to be greater in diabetic-derived cardiomyocytes with respect to normal myocytes. Following epinephrine stimulation, one would expect to see a greater change in phosphorylase activation in the diabetic myocytes relative to the normal myocytes due to the synergistic effects of both increased intracellular calcium and cAMP acting at the level of phosphorylase kinase. Furthermore, as the cells are maintained in primary culture for longer times (24 hours), intracellular calcium concentrations in both normal and diabetic myocytes would be expected to be in a state of change as a consequence of primary culture. It follows that after maintaining these cells in culture for 24 hours, that both normal and diabetic myocytes could exhibit an enhanced phosphorylase activation response due to the increase in cAMP following epinephrine stimulation and even greater intracellular calcium concentrations working in concert to influence phosphorylase kinase and hence phosphorylase activation. Conclusive proof of this hypothesis will require more definitive study.

Table 2

Comparison of phosphorylase b sources for use as a substrate to assay phosphorylase kinase activity

Source	-Phos b (cpm ± SEM)	+ Phos b (cpm ± SEM)	Difference
Phosphorylase b (Sigma)			
Control	145 ± 7.4	1295 ± 74	1150
- Epi	3294 ± 224	6815 ± 115	3521
+ Epi	2985 ± 186	7520 ± 420	4535
Phosphorylase b (Dobson)			
Control	100 ± 5.3	365 ± 13	265
- Epi	3230 ± 130	6068 ± 131	2838
+ Epi	2802 ± 246	6688 ± 219	3386
Phosphorylase b (Boehringer Mannheim)			
Control	87 ± 6.9	540 ± 14	453
- Epi	2435 ± 18	3137 ± 147	702
+ Epi	2892 ± 229	3614 ± 80	722

Normal hearts were perfused without (-Epi) or with 10 μM epinephrine (+Epi) prior to freezing in liquid nitrogen. Heart cytosolic extracts were prepared and [^{32}P] incorporation into phosphorylase b was measured in the presence of protein extract or homogenization buffer which served as the control. The data are expressed as the mean ± S.E.M. where n = 3.

CHAPTER VII

Summary and General Discussion

The primary focus of these studies was to characterize the molecular basis for the hypersensitive response of glycogen phosphorylase to catecholamine stimulation in primary culture, diabetic, rat ventricular cardiomyocytes. Toward this goal, several key regulatory aspects which could promote the hypersensitive response of phosphorylase, were investigated.

Initial studies indicated that rat cardiomyocytes derived from alloxan-diabetic animals exhibited a hypersensitive phosphorylase activation response to epinephrine stimulation relative to cells obtained from normal animals, which was apparent after 3 hours in primary culture and was further enhanced after maintenance of the cells in culture for 24 hours. While normal myocytes did not exhibit the hypersensitive response to epinephrine after 3 hours in culture, the response was acquired by these cells *in vitro* after 24 hours in primary culture (**Figure 3**). Based on studies utilizing selective α- and β-adrenergic receptor agonists and antagonists, the hypersensitive response of glycogen phosphorylase to epinephrine stimulation was shown to be mediated solely through the activation of surface β-adrenergic receptors.

To determine whether the presence of fatty acid metabolites could affect catecholamine-stimulated phosphorylase activation, normal and acute, alloxan-diabetic derived cardiomyocytes were maintained in culture for either 3 or 24 hours in the presence of various fatty acid metabolites (**Chapter 4**).

Palmitoylcarnitine, carnitine or carnitine in the presence of insulin failed to alter phosphorylase activation in response to epinephrine. In addition, inhibition of carnitine palmitoyltransferase 1 (CPT-1) activity by etomoxir did not alter the enzyme's response to the hormone. Collectively, these data indicated that the presence of fatty acid metabolites does not affect epinephrine-stimulated phosphorylase activation in normal and acute, alloxan-diabetic derived cardiomyocytes.

To address whether the hypersensitive response of phosphorylase resulted from altered signal transduction through the β-adrenergic receptor system or from a post-receptor defect, normal and diabetic-derived cardiomyocytes were incubated with either cholera or pertussis toxin prior to hormonal stimulation. Pretreatment of both normal and diabetic myocytes with cholera toxin resulted in a potentiated response to epinephrine stimulation whereas pertussis toxin did not affect the activation of this signaling pathway (**Figures 16 & 17**). Subsequent challenge of normal and diabetic myocytes with forskolin exhibited a similar activation pattern to that obtained with epinephrine (**Figures 18 & 19**). These studies demonstrated that while Gs does mediate the hypersensitive activation of glycogen phosphorylase in response to epinephrine stimulation, the hypersensitive response which is observed in alloxan-diabetic myocytes and is acquired *in vitro* in normal myocytes principally results from a defect occurring at a post-receptor site.

Lastly, to assess the role of phosphorylase kinase in the hypersensitive activation of glycogen phosphorylase in the diabetic heart, phosphorylase kinase activity was measured (**Chapter 6**). These studies indicated that while measurement of the enzyme's specific activity at pH 6.8 is an accurate and

reliable method for determining the amount of active phosphorylase kinase activity in the whole heart, the assay conditions need to be refined before this method can be used for measuring phosphorylase kinase activity in primary culture cardiomyocytes.

While the precise mechanism for the hypersensitive response of cardiac phosphorylase in diabetic rats is still unidentified, the data presented in this thesis provides supporting evidence which localizes the phenomenon to a post-receptor site which occurs during the later stages of the phosphorylase activation cascade. Collectively, these studies demonstrate that the defect is not present at the level of the β-adrenergic receptor, the activation of the stimulatory G-protein Gs, or the activation of adenylyl cyclase. Earlier studies have demonstrated that cardiac cyclic AMP accumulation and the increase in cAMP-dependent protein kinase activity ratio following β-adrenergic stimulation are also unaffected by diabetes (64,79). Similarly, phosphorylase phosphatase activity, the enzyme which is responsible for dephosphorylating and inactivating phosphorylase a, was shown to be unaltered by either epinephrine or diabetes (64). Based on this information, it is reasonable to hypothesize that the hypersensitivity of glycogen phosphorylase activation in primary culture diabetic ventricular cardiomyocytes is due to an enhanced sensitivity of phosphorylase activation at the level of phosphorylase kinase.

There are three plausible mechanisms which could account for the enhanced activation of glycogen phosphorylase in the diabetic heart. First, if there is a decrease in the phosphatase activities which regulate phosphorylase kinase activity, the net result would be a sustained activation of the enzyme which would be reflected by an enhanced activity response at the level of

phosphorylase. Phosphorylase kinase is subject to dephosphorylation by not only protein phosphatase 1 but also by protein phosphatase 2A (21,23). While the type 1 protein phosphatase accounts for the majority of phosphorylase kinase activity (toward the β-subunit which plays the dominant role in the enzyme's activation), protein phosphatase 2A is the major enzyme which acts on the α'-subunit of phosphorylase kinase (21). How these enzymes function together *in vivo* to bring about the inactivation of phosphorylase kinase is a complex issue which has not been fully resolved. Therefore, it becomes difficult to speculate on the specific effects of diabetes on phosphorylase kinase phosphatase activity in the heart.

The second mechanism which could promote the hypersensitive activation of phosphorylase could be the presence of an inhibitory factor (or factors) *in vivo* which exists in the heart to suppress the enzyme's activation in response to hormonal stimuli. If this humoral mediator became altered with the onset of diabetes, such that it was either missing or inactivated, the enhanced activation of phosphorylase following epinephrine stimulation would be seen in whole hearts or primary culture cardiomyocytes derived from diabetic animals. The existence of a humoral factor which blunts phosphorylase activation in the heart while possible is not probable due to the observation that upon supplementation of 3 and 24 hour, normal cardiomyocyte cultures with serum (concentrations ranging from 0 to 100%), the activation of phosphorylase was not altered in response to epinephrine challenge (**Chapter 3**).

The third mechanism which could account for the hypersensitive activation of phosphorylase may be due, in part, to the allosteric effects of calcium on phosphorylase kinase activity. Several studies have documented abnormal

calcium metabolism in the diabetic heart with the net result being an increase in intracellular free calcium (1,31,43,67,88). As described earlier, cardiac phosphorylase kinase is regulated by cAMP-dependent protein kinase and the allosteric effects of calcium (18,84). While a change in the phosphorylation state of the enzyme, due to the action of the kinase, would be a stable, distinguishable change, the allosteric effect of calcium on phosphorylase kinase is not a stable change which cannot be easily detected or assayed (60).

One observation which is consistent with the role of calcium in the hypersensitive activation of phosphorylase is the observation that when cardiomyocytes are maintained in primary culture for 3 or 24 hours, basal phosphorylase activity is identical for normal and diabetic-derived cells (approximately 5% phosphorylase a). It is only when diabetic-derived or normal myocytes after 24 hours in culture, are subject to β-stimulation that they exhibit the hypersensitive phosphorylase response. Since both calcium and cAMP produce synergistic increases in the amount of active phosphorylase kinase which is present in the heart, it seems highly probable that increased intracellular free calcium is directly involved in promoting the hypersensitive activation of phosphorylase by virtue of its actions at the level of phosphorylase kinase.

Further work will be required to provide evidence to support this hypothesis. The implications that an abnormality in calcium metabolism is involved in mediating the adverse pathological effects of diabetes remains to be determined.

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