REGULATION OF ß-ADRENERGIC-INDUCED PROTEIN PHOSPHORYLATION IN THE MYOCARDIUM

A Dissertation

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Edward E. George

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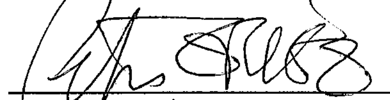
Approved as to style and content by:

Cheryl Scheid, Ph.D., Chairman of the Committee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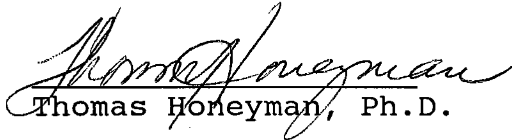
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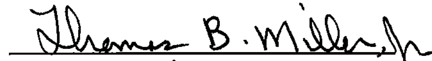
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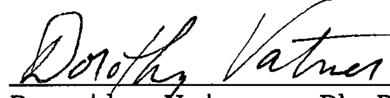
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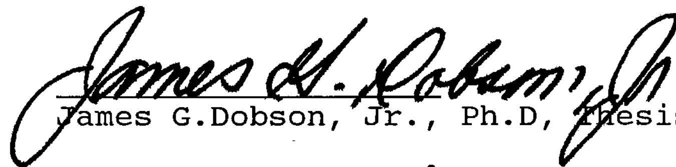
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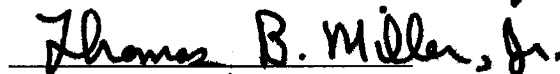
Thomas B. Miller, Jr., PhD., Dean

Graduate School of Biomedical Sciences

Department of Physiology

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DEDICATION

This work is dedicated to my wife, Jean, without whose support this would have been far more difficult and far less worthwhile.

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I would like to express my sincere gratitude to Dr. James Dobson for his continual support and guidance during my research.

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ABSTRACT

The purpose of this investigation was to examine selected biochemical mechanisms known to influence contractility and energy metabolism in the myocardium, with particular emphasis placed on the regulatory role of protein phosphorylation in the ventricular myocardium. The investigation was conducted in three phases; initially the cardiac contraction cycle was examined to determimine whether reported fluctuations in myocardial cAMP levels were associated with other biochemical events known to be cAMP-dependent. The second phase involved the determination of specific kinase activities and endogenous substrates in a highly purified cardiac sarcolemmal preparation. In the final phase, ventricular myocytes were utilized to examine the ability of adenosinergic and muscarinic agonists to influence the isoproterenol-induced increases in protein phosphorylation.

Studies in the first phase examined cyclic AMP levels and selected kinase activities in hearts frozen at various stages of the cardiac cycle. An automated clamping device, capable of freezing a perfused rat heart in less than 50 msec, was utilized to separate the cardiac cycle into various phases. Three different timing schemes were employed to divide the cycle into 2 to 4 segments. These different timing schemes revealed no significant differences in cAMP during the cardiac cycle. Myocardial

cAMP values ranged from 2.5 to 4.1 pmol/min/mg protein in all phases. However, in one scheme there was a tendency for cAMP to be elevated in early systole, with minimal values occurring diastole. There were also no significant differences seen for either glycogen phosphorylase or cAMP-dependent protein kinase (PKA) activity between various phases of the cardiac cycle. Since no significant fluctuations were observed in the levels of cAMP or the activities of PKA or glycogen phosphorylase during a single cardiac contraction cycle, it would appear that these agents do not exert their effects on cardiac function on a beat to beat basis.

The second phase of study examined the nature and function of individual protein kinases in the myocardium. Using a highly purified cardiac sarcolemmal preparation, kinase specific, synthetic substrates were employed to quantify the activities of cAMP-dependent (PKA), calcium/calmodulin-dependent (PKCM), calcium/phospholipid-dependent (PKC) and cGMP-dependent (PKG) protein kinases. Additionally, endogenous protein substrates were examined in this preparation to provide possible insight as to the function of these kinases in the heart. The activities of PKA, PKG, PKCM, and PKC in nmol ^{32}P/min/μg protein were as follows: PKA, 1606; PKG, 35.7; PKCM, 353; and PKC, 13.2. Three endogenous protein substrates of apparent molecular weights of 15kD, 28kD and 92kD were phosphorylated. While no endogenous protein

phosphorylation was detectable as a result of cG-PK activity, all of the substrates were phosphorylated, to varying degrees, by both PKA and CACM-PK. PKC phosphorylated only the 15kD substrate.

Even though several endogenous kinases are evident in the sarcolemmal preparation, cAMP-dependent protein kinase demonstrates the greatest degree of activity. This kinase also appeared to be the most abundant; however, there is some concern as to the source of these kinases in the membrane preparation since endothelial membranes as well as cardiac membranes appeared to be present. Evidence for endothelial contamination was provided by the finding that the membrane preparation contained appreciable amounts of angiotensin converting enzyme (ACE) activity, an enzyme felt to reside in the vascular endothelium. Since studies with this preparation could not exclude contribution of nonmuscle cell membranes a model consisting solely of dispersed ventricular myocytes was developed.

The third phase of these studies examined protein phosphorylation in primary cultures of ventricular myocytes. Specifically, these studies examined protein phosphorylation induced by exposure to isoproterenol (ISO), a catecholamine known to effect changes in the phosphorylation state of proteins in the heart by means of a ß-adrenergic-mediated/cAMP-dependent mechanism was examined. Additionally, the effects of phenylisopropy-

ladenosine (PIA) and carbamyl choline chloride (CARB) were examined with regard to their anti-adrenergic role(s) in this process.

Adherent, collagenase-dispersed, radiolabelled (^{32}P) ventricular myocytes exposed to ISO demonstrated a dose and time dependent increase in ^{32}P incorporation into several endogenous protein substrates. When the myocytes were exposed (60 sec) to either PIA or CARB prior to the exposure to ISO, ISO-induced ^{32}P incorporation into protein substrates of apparent molecular weight of 6kD, 31kD and 155kD was reduced up to 67% when compared to the effects of ISO alone. Additionally, both PIA and CARB attenuated the ISO-induced increase in PKA activity in the myocyte, yet only CARB was seen to produce an inhibitory effect on the ISO-induced increase in cAMP levels in the myocytes. The effects of CARB were dose-dependent and inhibited the effects of ISO on ^{32}P incorporation at all doses tested. PIA elicited biphasic effects: lower PIA concentrations were inhibitory in nature, while higher concentrations of PIA appeared to potentiate the increase in ^{32}P incorporation induced by ISO. Based on electrophoretic mobilities (SDS/PAGE) of the 6kD and the 155kD substrates, these substrates have been tentatively identified as the monomeric form of the sarcoplasmic reticulum-associated protein, phospholamban, and the contractile filament-associated protein, C protein, respectively. The 31kD substrate has been identified, by means of immuno-

blot, as the contractile filament-associated protein, troponin I.

The role of protein phosphorylation in the myocardium involves complex, inter-related mechanisms that encompass extracellular, transmembranal and cytoplasmic elements in the heart. It is well understood that certain mechanisms of the contraction cycle known to vary on a beat to beat basis, such as myosin ATPase, involve changes in protein phosphorylation. However, the nature of the various kinases and substrates examined in this study appear to influence longer-term events of myocardial contractility. Mechanisms coupled with hormone action, modulation of second messenger-dependent components, and factors associated with changes in contractility seen with aging and disease are more likely to exhibit changes similar to those described herein. A better understanding of the underlying biochemistry may provide greater insight into the importance of these metabolic changes.

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CHAPTER I

INTRODUCTION

The unique, all or nothing, manner of myocardial contraction dictates that the regulation of contractile function in the heart relies primarily on internal biochemical mechanisms. An integral component of these mechanisms is the close control and coordination of the intracellular levels of calcium and cAMP, as well as the activity of protein kinases and the phosphorylation state of several proteins in the ventricular myocyte.

The cyclic oscillation of certain events in the myocyte is suggested by the regular and repetitive nature of cardiac contraction. Although the variation in calcium levels over a single contraction cycle in the myocyte has been well established (83), evidence for the cyclic variation of additional elements is not as distinct. It has been suggested that the intracellular levels of cAMP, ATP, creatine phosphate, glucose-6-phosphate, and lactate, as well as the activity of the enzyme, glycogen phosphorylase, vary over the course of a single contraction cycle (141). However, the rigorous confirmation of these variations are, to date, somewhat limited by available experimemtal techniques necessary to directly assess these variables.

Extrinsic control of the heart is provided by the

autonomic nervous system, with ß-adrenergic (positive) and cholinergic (negative) neurotransmitters influencing contractile state. These extrinsic factors are involved more with the myocardial response to stress. In contrast, intrinsic control mechanisms, generally described by Starling's Law, are primarily concerned with the basal activity of the heart.

Given the unique manner of contraction in the myocardium and the apparent roles of protein phosphorylation and the intracellular concentrations of cAMP and calcium to this process, the following questions were proposed for investigation.

1. Can we detect fluctuations in intracellular cAMP and/or in cAMP-dependent processes during a single contraction cycle in the perfused heart?
2. What are the relative activities of cAMP-dependent, cGMP-dependent, calcium/calmodulin-dependent and calcium/phospholipid-dependent protein kinases in the sarcolemma, and what are their endogenous protein substrates?
3. Since cholinergic and adenosinergic agonists are known to antagonize the positive inotropic effects of adrenergic agonists in the heart, can we explain the antagonistic actions of these agents at the level of cAMP production, activation of PKA and/or changes in the phosphorylation of protein substrates in ventricular myocytes?.pa

While the fiber length of the myocardium is the primary determinant of the strength of contraction of the heart, sympathetic stimulation can enhance contractile state. This manner of stimulation occurs predominantly by activation of myocardial ß-adrenergic receptors. Early investigations in the heart and other tissues demonstrated that cAMP levels increased in response to exposure to catecholamines (128,129). Although phosphorylation was known to regulate the activity of glycogen synthase and phosphorylase kinase (39,80), the link between cAMP elevation and protein kinase activation was only established by the demonstration of a cAMP-dependent protein kinase (137). With the subsequent demonstration of the role of troponin and myosin as substrates for protein kinases (104,126), the significance of protein phosphorylation for regulation of cardiac function was established.

Concomitant with the emergence of the critical role of protein phosphorylation in the heart was the appreciation of the second messenger roles of cAMP and calcium (110). The potential feedback mechanisms in the control of calcium and cAMP, and the appreciation of specific events dependent upon these agents, suggests an interaction between regulatory pathways involving these second messengers.

Contraction in the ventricular myocyte is dependent upon the level of intracellular calcium. As the wave of depolarization reaches the myocyte during excitation-

contraction coupling in the myocardium, calcium flows into the cell, in turn triggering the release of additional calcium stored in the sarcoplasmic reticulum. In this calcium-induced calcium release process intracellular calcium concentrations are raised from diastolic levels of less than 10^{-7}M to levels on the order of 10^{-5}M (35). Calcium is then available to bind to a subunit of troponin (TNC), an action that relieves the inhibition of tropomyosin by troponin. This permits crossbridge formation between actin and myosin and the activation of a myosin ATPase, with the subsequent shortening of the muscle fiber (83). Relaxation at the level of the individual myocyte involves the reduction of intracellular calcium levels by resequestration into the sarcoplasmic reticulum and extrusion into the extracellular space (113). This reduction is brought about by calcium pumps in the sarcolemma and in the sarcoplasmic reticulum.

The interdependent nature of contractile regulation upon these second messengers is evident since the movement of calcium during the contraction cycle is modulated by cAMP-dependent mechanisms (136) and since phosphorylase kinase, a cAMP-dependent enzyme involved in glycogenolysis, has a distinct requirement for calcium (96). With an appreciation of the link between glycogen metabolism and cardiac contraction (54), and the role of protein phosphorylation in modulating these events, the mechanisms involved in controlling cellular levels of these second

messengers has been the focus of a large body of investigation in the field of cardiovascular physiology.

Signaling Pathway for ß-Adrenergic Agents. Membrane associated adenylyl cyclase catalyses the conversion of ATP to cAMP in the myocyte (35). This cytoplasmic face enzyme system is linked to the extracellular compartment via a transmembrane signaling system and is responsive to circulating catecholamines. The ß-adrenergic receptor is coupled to adenylyl cyclase through the GTP-binding protein, G_s (42). Also associated with this complex is another group of GTP-binding proteins, designated G_i, which serves to inhibit cyclase activity when acetylcholine and adenosine bind to muscarinic (M_2) and adenosinergic (A_1), respectively (55,116). When elevated, cAMP serves to activate membrane-associated kinases as well as soluble enzymes (14). The degradation of cAMP occurs via hydrolysis by phosphodiesterases (PDE). Several types of phosphodiesterases exist in the myocyte with sensitivities to calcium/calmodulin or cGMP (95). Thus, the increase in calcium in response to ß-adrenergic stimulation activates phosphodiesterase to limit the action of cyclic nucleotides.

Intracellular Domains and Protein Phosphorylation Sarcolemma. Inotropic state in the heart is most likely a function of calcium levels in the myocyte, with both myofibrillar sensitivity and myofibrillar exposure to the

calcium playing significant roles. Since free calcium levels in the cytoplasm range from 10^{-7}M to 10^{-5}M, while extracellular levels of the ion are near 10^{-3}M (35), it is apparent that the sarcolemma must play an important role in the control of calcium flux in the myocyte. There is a considerable burden on the cell to maintain proper calcium levels. This is evidenced by the sizable extracellular to intracellular concentration gradient, coupled with the large change in cytoplasmic calcium concentration during the contraction cycle. From diastole to systole the increase from 10^{-7}M to 10^{-5}M is due to an influx of calcium via the slow calcium channels in the sarcolemma and a release of calcium from the sarcoplasmic reticulum (30). At the level of the sarcolemma, this calcium burden is cleared using two different mechanisms. The majority of the calcium is removed from the myocyte by means of a sodium-calcium exchanger (Na^{+}-Ca^{2+} antiporter) resident in the sarcolemma. Calcium is also removed from the myocyte by an ATP dependent calcium pump. With the greater sensitivity for calcium shown by the calcium pump, it is felt that diastolic levels of calcium are maintained by this system. The antiporter is felt to be operative during systole (113). It has been reported that calcium influx through the slow channel is enhanced by cAMP-dependent protein kinase (102), presumably by phosphorylation of a channel-associated protein. Additionally, calcium or cAMP-dependent phosphorylation is felt to

enhance the velocity (V_{max}) of the ATP-dependent calcium pump, with no apparent effect on the Na^+-Ca^{2+} antiporter (15). Additional support for the role of protein kinase and phosphorylation in the regulation of calcium flux is provided by the catecholamine-enhanced increase in slow inward channel activity being reduced in the presence of muscarinic (132) or adenosinergic (120) agonists. The antiadrenergic actions of these agents are associated with reduction in cAMP-dependent protein kinase activity (21) and the reduction in the phosphorylation of protein substrates (36). Therefore, at the level of the sarcolemma, both calcium and cAMP-dependent processes may be involved in the mechanisms associated with an increase in inotropic state.

Sarcoplasmic Reticulum. Coupled with extracellular calcium as a source for increasing cytoplasmic levels of the ion is the calcium stored in the sarcoplasmic reticulum. This network of tubules surrounds the myofibrils of the sarcomere and is in contact with the invaginations of the sarcolemma making up the T tubule system (130). It is felt that the initial calcium flux triggered by the depolarization of the myocyte induces the release of the calcium stored in the sarcoplasmic reticulum, bathing the myofibrils in calcium and promoting contraction by modifying the interaction between the regulatory components (troponin) of the contractile proteins (127). During

relaxation, the calcium is resequestered into the sarcoplasmic reticulum by active transport via another Ca^{2+}-Mg^{2+}-ATPase (130). Phospholamban, a protein associated with the ATPase of the sarcoplasmic reticulum, has been shown to be phosphorylated by cAMP-dependent protein kinase and calcium/calmodulin-dependent protein kinase (139). The increase in time to peak tension development and rate of relaxation produced by isoproterenol in the heart may be correlated with phospholamban phosphorylation (86). An increase in phosphate incorporation into phospholamban by cAMP-dependent protein kinase corresponds with an increase in calcium transport into the sarcoplasmic reticulum (76) which can effect both the rate of calcium removal and the amount of calcium stored. Thus, the increased rate of relaxation produced by isoproterenol stimulation may be due to the more rapid resequestration of calcium into the sarcoplasmic reticulum caused by the increased phosphorylation state of phospholamban; and the increased rate of tension development may be a result of the greater release of calcium from the sarcoplasmic reticulum.

Contractile Proteins. Calcium produces a change in the interaction of the contractile proteins, myosin and actin, and the regulatory proteins associated with these elements in the sarcomere. While the light chain subunits of myosin appear to be substrates for calcium/calmodulin-dependent protein kinase in vitro, no apparent physiologi-

cal role for this phosphorylation has been demonstrated in cardiac muscle (60). C-protein, a protein associated with the myosin complex, has been shown to be a substrate for cAMP-dependent protein kinase; and, the change in phosphorylation of this protein correlates with an increase in myofibrillar Ca^{2+}-ATPase activity and an increase in inotropic state. The dephosphorylation of this protein does not correspond temporally with the removal of catecholamine stimulation (47); Thus the function of this protein is unclear. Other proteins associated with the sarcomere may be modulated by phosphorylation. For example, the troponin complex (troponin I, T, C) is involved with the tropomyosin molecule in the regulation of actin and myosin interaction (127). Troponin C possesses three calcium binding sites, two being high affinity sites that are occupied at the low calcium concentrations seen in diastole. As the calcium concentration increases during systole, the third, low affinity calcium site on troponin C becomes occupied, increases the affinity of troponin C for troponin I and induces a steric shift in the molecules exposing the myosin binding site on the actin filament, thereby permitting interaction with the myosin filament. With actin and myosin able to more freely interact, contraction occurs. Troponin I has been shown to be a substrate for cAMP-dependent protein kinase (32). This increase in phosphorylation state is associated with a decrease in sensitivity for myofibrillar calcium binding

(111). And it has been postulated that this alteration in calcium affinity is responsible for the increased rate of relaxation seen with catecholamine stimulation (70).

Glycogen Particle. The contractile process relies on precise metabolic control to provide the requisite energy for contraction. An important source of energy in the myocyte is glycogen and glycogenolysis is regulated by a series of multiple and interdependent enzymatic reactions (54).

ß-adrenergic stimulation leads to an activation of glycolysis in 2 ways: 1. by stimulating phosphorylation of phosphorylase kinase, a process mediated by ß-adrenergic activation of adenylyl cyclase, increased levels of cAMP and increased activity cAMP-dependent protein kinase and 2. increased calcium availability, a process involving ß-adrenergic actions to increase calcium stored and increase calcium channel activity (94).

The role of protein phosphorylation is evident in the precise coordination within the cascade of cAMP-dependent events. The activation of glycogen phosphorylase by phosphorylation is accompanied by the concomitant inactivation of glycogen synthase by phosphorylation; and the control of opposing metabolic processes is achieved by one signaling mechanism.

Control of Phosphorylation. Protein kinases associated with the majority of phosphorylations taking place in the