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Regulation of β-Adrenergic-Induced Protein Phosphorylation in the Myocardium: A Dissertation

Edward E. George

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

A Dissertation

By

Edward E. George

Submitted to the Faculty of the University of Massachusetts Graduate School of Biomedical Sciences, Worcester in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY IN MEDICAL SCIENCES

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PHYSIOLOGY
REGULATION OF β-ADRENERGIC-INDUCED PROTEIN
PHOSPHORYLATION IN THE MYOCARDIUM

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October 1990
DEDICATION

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

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

An additional thanks goes to the members of my research committee, Drs. Honeyman, Miller and Scheid for their interest and expertise and to Dr. Steve Jasper for all his help with those cells.

I owe a special debt of gratitude to my coworkers in the Dobson group for always lending a hand. And thanks to Drs. Peter Grigg and Fred Romano for their outstanding senses of humor.
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 determine 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 β-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 experimental techniques necessary to directly assess these variables.

Extrinsic control of the heart is provided by the
autonomic nervous system, with β-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?
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 β-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 $10^{-7} \text{M}$ to levels on the order of $10^{-5} \text{M}$ (35).
Calcium is then available to bind to a subunit of troponin
(TNC), an action that relieves the inhibition of tropomyo-
osin 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 extru-
sion 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 glycogenoly-
sis, 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 β-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 β-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 β-adrenergic stimulation activates phosphodiesterase to limit the action of cyclic nucleotides.

**Intracellular Domains and Protein Phosphorylation Sarco-lemma.** 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 \(10^{-7}\text{M}\) to \(10^{-5}\text{M}\), while extracellular levels of the ion are near \(10^{-3}\text{M}\) (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 \(10^{-7}\text{M}\) to \(10^{-5}\text{M}\) 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 (\(\text{Na}^+-\text{Ca}^{2+}\) 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 \( V_{\text{max}} \) 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\textsuperscript{2+}-Mg\textsuperscript{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\textsuperscript{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).

β-adrenergic stimulation leads to an activation of glycolysis in 2 ways: 1. by stimulating phosphorylation of phosphorylase kinase, a process mediated by β-adrenergic activation of adenylyl cyclase, increased levels of cAMP and increased activity cAMP-dependent protein kinase and 2. increased calcium availability, a process involving β-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
heart may be categorized as cyclic nucleotide-dependent or calcium-dependent. Most of the actions of catecholamines in the heart are mediated by cAMP-dependent protein kinase (PKA). This enzyme exist as in two isoforms in the heart, with the predominant type varying with species, however there are no apparent differences in the substrate specificity for the two isoforms (49). PKA exists in both the soluble and particulate fractions of the heart, with half maximal activation of the enzyme occurring at a cAMP concentration of 1.0 μM (48). A second cyclic nucleotide-dependent protein kinase, cGMP-dependent protein kinase, is activated by muscarinic stimulation of the heart. Substrates unique to cGMP-dependent protein kinase have not been identified; and although able to phosphorylate substrates of PKA, the kinetic parameters of these enzymes are such that it is unlikely that phosphorylation of these substrates by cGMP-dependent protein kinase occurs in vivo (61).

Calcium-dependent protein kinases may be separated into two categories: calcium/calmodulin-dependent and calcium/phospholipid-dependent. Phosphorylase kinase, a key enzyme in the glycogenolytic pathway, is activated by calcium. Although phosphorylation of this enzyme by PKA slightly increases its affinity for calcium, it is the binding of calcium to the calmodulin subunit of the enzyme that activates this kinase. Once activated, this kinase converts glycogen phosphorylase to by phosphorylation
Myosin light chain kinase (MLCK) is also activated by its formation of a ternary complex with calcium and calmodulin. In smooth muscle, MLCK increases myosin ATPase activity; however in the heart, myosin light chain phosphorylation does not affect myosin ATPase activity and its function is at present unclear (59). Initially isolated in the brain (131), calcium/phospholipid-dependent protein kinase (PKC) has been characterized at low levels in the heart (81). PKC, in common with other kinases, phosphorylates a number of substrates such as troponin I, troponin C, myosin light chains, phospholamban and several sarcolemmal proteins. In addition this enzyme phosphorylates several unique substrates (5). Although a clear role for PKC has not been identified, it may function in a modulatory manner with regard to the actions of other enzymes.

Since covalent modification by protein phosphorylation is considered to provide a regulatory function, a counteracting mechanism(s) must exist to modulate this type of control process. Phosphoprotein phosphatases have been associated with the dephosphorylation of various substrates in the heart. Protein phosphatase-1 and -2 are associated with glycogen metabolism, with phosphatase-1 associated with the glycogen particle and responsible for approximately 80% of the phosphatase activity. Phosphatase 1 dephosphorylates glycogen phosphorylase, glycogen synthase and phosphorylase kinase, thereby promoting a
down regulation of glycogenolysis (19). Phosphatase inhibitor proteins (IP1 and IP2) regulate the activity of phosphatase-1 (96). The phosphorylation of IP1 by PKA results in an inhibition of phosphatase-1 activity and demonstrates the high degree of coordination between events involved in the energy metabolism of the heart. Phosphatases involved in the dephosphorylation of myofibrillar proteins are less well characterized. Myosin light chain phosphatase, as well as the phosphatase(s) associated with the dephosphorylation of troponin I have been characterized only to the extent that they vary in reaction kinetics and type of response to hormonal stimulation (99).

Regulation of Contractility and Glycogenolysis. β-adrenergic agents have been shown to produce marked increases in inotropic state and glycogenolytic activity (8) in the heart. Through the elevation of cAMP and the increased activity of PKA, the β-adrenergic challenged heart increases cytosolic calcium levels during systole, more rapidly eliminates calcium during diastole (83) and increases the rate of glycogen breakdown (13) to fuel the higher metabolic demands of the heart under stress.

The role of protein phosphorylation in the control of the inter-related events during β-adrenergic stimulation of the heart involves both the activation and inhibition of several processes. While troponin I, phospholamban
and a 15 kD protein associated with the slow inward calcium channel are phosphorylated to enhance contraction (77), glycogen synthase and a phosphoprotein phosphatase inhibitor protein are phosphorylated to inhibit their respective roles in glycogen synthesis and dephosphorylation of certain proteins (100). Additionally, the calcium sensitivity of myosin ATPase and phosphorylase kinase are regulated by cAMP-dependent phosphorylation (40).

**Agonist Specificity.** However, other agents that act by increasing cAMP levels in the heart and by activating cAMP-dependent protein kinase do not always produce the changes in contractile state associated with β-adrenergic stimulation. The diterpene, forskolin, and prostaglandin E₁ (PGE₁) both elevate cAMP levels and PKA activity in the heart (13). The elevation of cAMP levels in the perfused heart caused by forskolin are 2 to 3 times greater than the elevation produced by the β-adrenergic agonist, isoproterenol, yet the changes in contractility (+dP/dt) and in PKA activity were similar to those changes seen with isoproterenol. PGE₁ produced no change in contractile state despite producing an increase in cAMP and PKA activity in the perfused heart (52). Examination of intracellular compartments by fractionation studies, indicates that the cAMP elevation associated with the particulate component of the cell is required to activate a membrane associated PKA, which then dissociates to the cytoplasm (53). It has been proposed that β-adrenergic agents
preferentially activate this subcellular component of PKA, which has access to specific co-factors or substrates associated with increased contractility (14).

**Antiadrenergic Effects in the Heart.** While adrenergic stimulation of the heart is the predominant method of increasing inotropic state, cholinergic stimulation produces an inhibitory or antiadrenergic effect on the catecholamine stimulated heart (56). Adenosine also inhibits β-adrenergic actions in a manner that closely resembles the antiadrenergic effects of acetylcholine (22). The effects of both of these antiadrenergic agents may be seen as a reduction in pressure generation, a decrease in the catecholamine-enhanced activity of several enzymes in the glycolytic pathway, a reduction in the catecholamine-induced phosphorylation of protein substrates and a decrease in the catecholamine-stimulated levels of adenylyl cyclase activity and cAMP production (36, 47, 88).

The significance of acetylcholine and adenosine effects may in part be related to their continued presence in the myocardium. Acetylcholine is continually released by vagal nerves to the heart. Adenosine, present as a metabolite in the intracellular and extracellular compartment is continually produced. Additionally, adenosine levels increase in response to ischemia, hypoxia and the presence of endogenous catecholamines in the myocardium (36). Acetylcholine and adenosine reduce the electrophys-
iologic changes associated with catecholamine stimulation of the heart (140).

**Antiadrenergic Mechanisms.** The manner by which these antiadrenergic agents attenuate catecholamine-induced events in the heart is unclear. However it appears that both muscarinic and adenosinergic receptors that are inhibitory with respect to adenylyl cyclase in the heart are coupled to the enzyme by an inhibitory version of the nucleotide binding protein, G_i (115). This is supported by the effects of both these antiadrenergic agents being sensitive to pertussis toxin (55). Therefore, antiadrenergic agents may provide an inhibitory signal to the cyclase at the same time that β-agonists are providing a stimulatory signal, with the balance being a reduced signal. In addition to the inhibitory A_1 adenosinergic receptor, there exists an A_2 receptor which is stimulatory toward adenylyl cyclase (116). This receptor, while less sensitive to adenosine than the A_1 receptor will enhance adenylyl cyclase activity in the heart at adenosine concentrations above micromolar (22).

Phosphatase activity is also modulated. These enzymes reverse the actions of adrenergic agents stimulation, and act to limit the influence of β-adrenergic agents on inotropic state (28). Thus a neurotransmitter, acetylcholine, and a local metabolite, adenosine, both
produce similar effects using common and different pathways.

**Metabolic Oscillations During the Contraction Cycle**. The cyclic nature of events associated with cardiac contraction suggests oscillation(s) of certain metabolic events. The level of intracellular calcium increases from diastole to systole through an increased inflow through voltage sensitive calcium channels, coupled with the release of calcium from the sarcoplasmic reticulum. Calcium levels are returned to diastolic concentration by the actions of calcium ATPases on the sarcolemma and the sarcoplasmic reticulum. Elements of the contractile apparatus, such as troponin and myosin ATPase, appear to vary in activity as a function of the cross-bridge cycle (118). Additionally, compounds involved in metabolic pathways, such as ATP, glucose-6-phosphate, lactate and creatine phosphate, as well as cyclic nucleotides (cAMP and cGMP) and glycogen phosphorylase have been reported to vary in concentration during the cardiac cycle (38,141).

Some of the oscillations that occur during a cardiac cycle, such as the variability in calcium flux through the slow channels, are evident. However, other potential cyclic oscillations such as with cAMP, have not been clearly demonstrated. The role of intracellular compartmentalization has been offered in explanation for activation of some but not all steps in a pathway, such as with the cAMP-dependent cascade [ ]. However, evidence for such
compartments, although attractive, is not readily available. Additionally, the role of protein phosphorylation in this process in the heart is not completely clear. The phosphorylation of certain proteins can be directly correlated to contractile related events, yet many of the phosphorylations occurring with β-adrenergic stimulation of the heart have not been ascribed a physiological role.

Modulation of Contractile State. Since the contractile state of the myocardium is dependent upon the concentration of calcium available to the myofilaments, as well as the sensitivity of the myofilaments to calcium, mechanisms affecting the availability of calcium would be likely sites for inotropic modulation. With the slow inward calcium channel providing entry of calcium into the cell, to the point of triggering the release of calcium by the sarcoplasmic reticulum (35), the sarcolemma can be viewed as an initial level of control. β-adrenergic stimulation results in an increase in the calcium slow current (31,72). A protein that co-purifies with the slow-inward calcium channel is phosphorylated by β-adrenergic activation of cAMP-dependent protein kinase and is believed to influence the slow channel in a manner to increase calcium flux (124). Cholinergic agonists, known to attenuate β-adrenergic effects in the heart, reduce both the adrenergic stimulation of the slow channel and the possible phosphorylation of this "channel-associated" protein
To expel the calcium accumulated in the sarcoplasm during systole, the sarcolemma utilizes the sodium/calcium exchanger and a high affinity, low capacity calcium/ATPase (16). The bulk of the calcium expelled from the myocyte is due to the exchanger, a process not felt to be regulated by phosphorylation. However, the calcium/ATPase may display an increased $V_{\text{max}}$ upon phosphorylation by either cAMP-dependent or calcium-dependent kinases (57).

The release of calcium from the sarcoplasmic reticulum is triggered by the increased calcium entering the myocyte through the slow inward channel. While phosphorylation does not directly alter elements of the sarcoplasmic reticulum involved in calcium release, cAMP-dependent phosphorylation of phospholamban can impact the sarcoplasmic reticulum by altering the reuptake of calcium (76). This phosphorylation correlates with the increased rate of relaxation seen with $\beta$-adrenergic stimulation (124) and is consistent with the greater calcium release by the sarcoplasmic reticulum during $\beta$-adrenergic stimulation. Since phospholamban is closely associated with the calcium/ATPase of the sarcoplasmic reticulum, phospholamban phosphorylation may influence the activity of this ATPase. Both the rate of calcium resequestration and the degree of protein phosphorylation are attenuated by anti-adrenergic agents such as acetylcholine (63).

The contractile apparatus of the sarcomere is subject to modulation by phosphorylation at both the level of
the contractile and regulatory proteins (66). A light chain associated with the head region of the myosin filament is a substrate for calcium/calmodulin-dependent myosin light chain kinase; however the physiological role of this phosphorylation is unclear (60). C-protein, a 150,000 kD protein postulated to have structural significance in the junctional region between the head and the tail regions of the myosin filament (46), is a substrate for cAMP-dependent protein kinase and is phosphorylated in the heart following β-adrenergic stimulation (40).

At the level of the thin filament, regulation of the troponin-tropomyosin complex may involve phosphorylation of several proteins. The normal inhibitory role of troponin I regarding the calcium affinity of troponin C is enhanced by phosphorylation (111). Since the phosphorylation of troponin I correlates with the increase in inotropic state seen during β-adrenergic stimulation, it is speculated that the decrease in troponin C affinity for calcium caused by TNI phosphorylation may underlie the increased rate of relaxation (70).

Experimental Models. Most investigation into cardiac metabolism has been performed on the perfused heart. This model offers the ability to correlate a change in contractile function to a change in a biochemical event. However, the perfused heart as an experimental model poses problems related to the heterogeneity of cell types present in the preparation, coupled with the high levels
of isotope required for examination of phosphorylation related investigations. The problem of one heart providing only one experiment further compromises the perfused heart as a model.

**Isolated Ventricular Myocytes.** Over the past decade the use of isolated cardiac myocytes has provided the ability to examine metabolic events in the cardiac cells directly. The isolated cells permit multiple experiments from one heart, the use of lower amounts of radioactivity than with the perfused heart model and precise control of experimental conditions (142).

Cells are dispersed using several different treatments in which varying amounts of collagenase and hyaluronidase and other compounds are included. These myocytes may then be employed in one of 3 ways, as freshly dispersed cells in suspension, as primary cultures or as long term cultures. Since myocytes consist of both viable cells and damaged cells the results from studies using freshly dispersed cells in suspension may be compromised by the response of damaged cells. Long term culture of myocytes provides the ability to perform chronic studies, however several weeks in culture are required for the necessary transformation or redifferentiation to a cell that metabolically resembles a cardiac myocyte (64). On the other hand the primary cultured myocyte retains the morphology of the striated cardiomyocyte and exhibits
pharmacological and contractile behavior similar to the whole organ.

The contractility of the isolated myocyte has been examined under different experimental conditions. Increases in extracellular calcium produce an increase in the rate and frequency of spontaneous contraction, while field stimulation produces a reversible contraction of the myocyte (75). β-adrenergic stimulation of the isolated myocyte produces an increase in rate and frequency of contraction, changes in protein phosphorylation, enzyme and ion channel activities (98, 101).

β-adrenergic stimulation of the myocyte produces increases in the phosphorylation of several proteins associated with the increase in contractile state seen in the perfused heart exposed to β-adrenergic agents. Phosphorylation of phospholamban and TNI increases with β-adrenergic stimulation (101). Additionally, a protein associated with the slow inward calcium channel increases in phosphorylation under similar conditions (124). Enzymes associated with the cAMP-dependent cascade have been shown to respond in a manner corresponding to the enzymatic changes seen in the catecholamine challenged perfused heart (143). The presence of a β-adrenergic response suggests that the stimulatory G protein (Gs) mechanism(s) is functionally intact in the isolated cell.

In addition to a functional β-adrenergic receptor system, isolated cardiomyocytes have been shown to possess
functional muscarinic (145) and adenosinergic (58,116) receptors. Myocytes also respond physiologically to forskolin, insulin, glucagon and histamine (8). The numerous functional receptors, coupled with the appropriate physiological response, support the isolated cardiomyocyte as a viable model for the examination of myocardial metabolism.

Since the cellular response to β-adrenergic agents parallels the responses seen in the intact heart, it is interesting to note that the anti-adrenergic effects of muscarinic and adenosinergic agents are also seen in the isolated cardiomyocyte (58,116). These anti-adrenergic effects suggest the presence of a functional inhibitory G protein (G₁). Furthermore, while acetylcholine produces a decrease in cAMP levels and an increase in IP₃ in myocytes exposed to β-adrenergic agents (85), adenosine only affects cAMP levels (84). These responses, again similar to those seen in the intact organ, imply that the mechanisms differentiating these effects are also present in the isolated cell.

In the intact heart the anti-adrenergic effects of adenosine and acetylcholine are associated with an attenuation of adenylyl cyclase activity and cAMP-dependent events such as protein kinase activity and protein phosphorylation (124). In the isolated myocyte, less is known concerning anti-adrenergic effects involving adenosine and acetylcholine. However, the present studies demonstrated
that adenosine produces inhibitory effects at low concentrations and stimulatory effects at elevated concentrations. Therefore the presence of the inhibitory (A1) and the stimulatory (A2) adenosine receptor is suggested in the isolated myocyte (116).

In examining myocardial metabolism, the cyclic manner of the cardiac contraction indicates a high degree of coordination and presupposes an efficient mechanism of recovery to avoid energy debt. Since the primary second messenger calcium is regulated in a precise and interdependent manner with cAMP, oscillations in events dependent upon these messengers may be considered. The sarcolemma, is associated with protein-kinase related activity and, appears integral to events involved with cardiac metabolism and contractility. While these intrinsic factors provide a degree of control in myocardial function, extrinsic factors, such as hormones, neurotransmitters and metabolites appear modulatory with regard to myocardial contractility and metabolism, with a primary means of control effected by covalent modification of proteins by phosphorylation.

In an effort to better understand the regulation of myocardial metabolism and contractility, the following studies were conducted. The cAMP levels were examined in the perfused heart to determine what fluctuations, if any, occurred during a single contraction cycle and whether these fluctuations were reflected in the appropriate
biochemical sequelae. The activity and protein substrates of several (cyclic nucleotide and calcium dependent) protein kinases were determined at the level of the sarcolemma. And the effects of the β-adrenergic agent, isoproterenol, as well as the anti-adrenergic agents adenosine and acetylcholine, were examined with regard to cAMP levels, cAMP-dependent protein kinase activity and the phosphorylation of select protein substrates.
CHAPTER II
LACK OF OSCILLATIONS IN CYCLIC AMP, CAMP-PROTEIN KINASE AND GLYCOGEN PHOSPHORYLASE DURING THE CARDIAC CYCLE IN THE PERFUSED RAT HEART

ABSTRACT
It is unclear whether reported fluctuations in the level of cyclic adenosine 3',5'-monophosphate (cAMP) during a single cardiac cycle in ventricular muscle are associated with distal changes in cAMP-dependent processes. The degree of cAMP variation and its effect, if any, on biochemical sequelae during the cardiac cycle, were investigated by determining the level of cAMP and the activity ratios of cAMP-dependent protein kinase and glycogen phosphorylase in the rat ventricular myocardium. Isolated perfused hearts contracting at 240 beats/min and free of exogenously administered catecholamines were freeze-clamped, utilizing an automated clamping device capable of freezing the entire heart in less than 50 msecs. The cardiac cycle was segmented into phases utilizing 3 different segmentation schemes. While no significant difference was detected between phases regardless of the method of segmentation for cAMP, cAMP-dependent protein kinase, or glycogen phosphorylase levels, there was an insignificant tendency for cAMP to be elevated during early systole in one method of segmentation. These results suggest that the levels of cAMP and the activities...
of cAMP-dependent protein kinase and glycogen phosphorylase do not vary significantly during a single cardiac cycle in the mammalian myocardium.
INTRODUCTION

Previous studies in amphibian and mammalian myocardium have reported oscillations in adenosine 3',5'-cyclic monophosphate (cAMP), guanosine 3',5'-cyclic monophosphate (cGMP), and the activity of cAMP-dependent protein kinase and glycogen phosphorylase that were thought to be associated with the contractile cycle (10,134,140). However, in several of these reports the presence of catecholamines appeared either to enhance or be required for the observed oscillations (10,78,141). Certain events such as the changes observed in calcium concentration and resultant interactions with troponin, as well as conformational changes in the relationship between actin and myosin over the course of a single cardiac cycle have been well described (34,67,70). Changes in ATP and creatine phosphate levels, utilizing gated nuclear magnetic resonance spectroscopy (8), and biochemical techniques (22) suggest that the levels of these compounds are highest during diastole, and lowest during systole. The levels of cAMP and the activity of cAMP-dependent protein kinase and glycogen phosphorylase have been reported to be maximal during systole and minimal during diastole (10,78,134,141). Conversely, the levels of cGMP have been reported to be minimal during systole and maximum during diastole (144). While it has been implied that these observed changes may be inter-related, little has been offered to describe these events with relation to the cardiac cycle in a
manner that demonstrates a cause and effect regarding the
cycle and its metabolic processes and requirements. For
example, the modest reported increase in cAMP during the
first half of systole (10,141,144) has not yet been directly
correlated with other events associated with myocardial
metabolism known to be cAMP-dependent in the mammalian
heart contracting at normal frequency and free of catecho-
lamine stimulation.

In an attempt to relate these observed fluctuations
in cAMP levels to the metabolism of the mammalian myocard-
dium, a device capable of rapidly freeze-clamping the
isolated perfused rat heart was used to examine the fluc-
tuations in cAMP levels during the contraction cycle in
the absence of exogenous catecholamine administration. In
addition, the importance of these fluctuations in terms of
influencing distal biochemical sequelae were assessed by
determining the activity ratios of cyclic AMP-dependent
protein kinase and glycogen phosphorylase.

METHODS

Rats used in this study were maintained and used in
accordance with recommendations in the Guide for the Care
and Use of Laboratory Animals, prepared by the Institute
of Laboratory Animal Resources, National Research Council,
U.S. Department of Health and Human Services, National
Institutes of Health Publication No. 85-23, rev. 1985 and
the Guidelines of the Animal Care Advisory Committee of
the University of Massachusetts Medical School.

Heart Perfusion. Isolated rat hearts were perfused ac-
cording to a method described previously (22). Briefly,
male Sprague-Dawley rats (300-350 g) were anesthetized and
heparinized (ip with a pentobarbitol: 40mg/kg and heparin: 500 Units. After approximately 20 minutes hearts were excised, briefly rinsed in ice-chilled physiological saline (PS) and immediately perfused at 8 ml/min with nonrecirculated PS at 37°C via an aortic cannula. The PS contained in mM amounts: 119.4 NaCl, 4.7 KCl, 1.2 MgSO₄, 2.5 CaCl₂, 10 glucose, 25 NaHCO₃ and 1.2 KH₂PO₄. The pH was maintained at 7.4 by gassing the PS with 95% O₂ - 5% CO₂. Hearts were paced with a Grass SD9 stimulator (5 msec duration and 10% above threshold) at 4 Hz using platinum electrodes attached to the right atrium. Left ventricular pressure was monitored with a saline-filled latex balloon-tipped canula inserted into the left ventri-
cle. Perfusion was continued for 15 minutes to allow each heart to equilibrate. Hearts that did not develop a maximum systolic pressure of 80 mm Hg at the end of the equilibration period were not used. The hearts had a rate of left ventricle pressure development that averaged 4230 % 96 mm Hg/sec, and increased to 7200 mm Hg/sec with a 30 sec perfusion of 10 nM isoproterenol. At various points during the contraction cycle, hearts were rapidly frozen
utilizing an automated freeze-clamping device described in detail by Thompson et al. (134). Briefly, this device utilizes the voltage signal from the pressure transducer as an index of the left ventricular pressure development. The variable threshold and delay parameters are preset in the logic controller of the device, such that the release of the spring loaded clamps is triggered at a predetermined point in the cardiac cycle. Precise resolution of the freezing point in the cycle was provided by the monitoring the transducer signal via a high speed chart recorder (Grass Model 7D polygraph) and a memory oscilloscope (Tektronix R5103N) to verify the exact time of freezing.

**Tissue Preparation.** Frozen hearts were pulverized in a chilled Waring blender containing liquid nitrogen and stored at -80°C prior to analysis according to methods described previously (24). All subsequent handling of the tissue and extracts occurred at either -20°C or in an ice/water slurry.

**Tissue Extraction and Analysis.** To measure the cAMP content of the myocardium 30-40 mg of powdered tissue was homogenized on ice in 0.5 ml of 10% trichloroacetic acid (TCA) using a Duall (Kontes, size 20) ground glass homogenizer. The homogenate was centrifuged at 3,000 x g for 2 mins (0°C), and the TCA was extracted from the supernatant 4 times with water-saturated ether. After permitting the
ether to evaporate at 80°C, samples were assayed for cAMP concentration using an $^{125}$I-cAMP RIA system (Amersham). This assay employs a cAMP-specific antibody, with the final separation being achieved by a second antibody bound to a polymer, thereby permitting separation of unbound labelled tracer by centrifugation. The cAMP values are reported in pmol/mg protein of original TCA homogenate.

Protein kinase activity of the myocardium was determined by homogenizing 20–30 mg of frozen powdered tissue in a Duall homogenizer containing 5 volumes of a solution containing 5 mM K$_2$HPO$_4$, 1 mM ethylenediaminetetraacetic acid, (EDTA) and 0.5 mM methyl-isobutylxanthine (pH 6.8) and centrifuging at 12,000 x g for 20 min (0°C). The supernatant extract was immediately assayed in the absence and presence of 2 mM cAMP for protein kinase activity, as described previously (20), after the method of Keely (69). The activity of the kinase is based on the phosphorylation of the histone (Type II-A, calf thymus) and one unit (U) of the enzyme is defined as the amount of enzyme that catalyses the transfer of 1 pmol of $^{32}$P from [$\gamma$-$^{32}$P]ATP to histone in 1 min at 30°C. Protein kinase activity is expressed as the percent activation which is the ratio of activity in the absence of cAMP to that in the presence of cAMP (2 mM) x 100. An increase in the percent activation ratio is assumed to reflect dissociation of the holoenzyme into its regulatory and active catalytic subunits. The total protein kinase activity is expressed as that enzyme
activity determined in the presence of 2 mM cAMP per mg protein in the supernatant.

Glycogen phosphorylase activity of the myocardial tissue was determined by homogenizing 100 mg of the frozen powdered tissue in 50 volumes of 100 mM KF, 50 mM 3-(N-morpholino)ethanesulphonic acid (MES), 15 mM β-mercaptoethanol (BME) and 5 mM EDTA (pH 6.8, 0°C) with a Polytron (Brinkman, PT10 probe; setting 5, 2 x 10 sec bursts). Norit charcoal (50 mg) was added to each sample for a 1 minute period prior to centrifugation at 8,000 x g for 10 min at 0°C to remove endogenous AMP. The supernatant was assayed for phosphorylase activity in the absence and presence of 2 mM AMP for both the formation of glucose-1-phosphate and glycogen. In the former, the formation of glucose-1-phosphate was determined after the method of Hardman (44), through the use of coupled enzyme reactions involving phosphoglucomutase and glucose-6-phosphate dehydrogenase. In the latter, the filter paper method of Gilboe (41) was utilized to measure the formation of $^{14}$C labelled glycogen. One unit of phosphorylase is defined as the amount of enzyme that either produces 1 mmol of glucose-1-phosphate or utilizes 1 mmol of glucose per min at 30°C. The results are expressed as the percent activation which is the ratio of phosphorylase activity without AMP to phosphorylase activity with AMP (2 mM) x 100. An increase in the percent activation indicates an increase in the conversion of phosphorylase b to a. The total
activity of the phosphorylase is expressed as that enzyme activity determined in the presence of 2 mM AMP per mg protein in the supernatant extract. All protein contents were determined after the method of Lowry (89).

Statistical Methods. All data are expressed as means ± one standard error of the mean. Statistical significance was determined using one way independent analysis of variance (122). A probability of less than 0.05 was accepted as indicating a significant difference.

Materials. All reagents were either of reagent or commercial grade. Calf thymus type II-A histone and 1-methyl-3-isobutylxanthine were obtained from Sigma Chemical. Radiolabeled compounds (32P)ATP and 14C-Glucose, as well as the cAMP radioimmunoassay kit, were obtained from Amersham Corporation. Nucleotides, glucose, glucose-phosphate and enzymes used for the phosphorylase assay were obtained from Boehringer-Mannheim.

RESULTS

A typical left ventricular pressure tracing obtained from an isolated beating rat heart is illustrated by the chart recorder tracing shown in Figure 1. The perfused hearts developed a mean systolic pressure of 95 mm Hg. Given the ability to freeze clamp a beating heart into a 1 mm thick wafer in less than 50 msec, at near liquid nitro-
gen temperature, it is estimated that a given sample will be completely reduced to 0°C in less than 10 msec.

FIGURE 1. A typical left ventricular pressure tracing of a perfused rat heart. Chart recorder tracing of analog pressure signal was obtained by a balloon-tipped cannula inserted into the left ventricle. The asterisked (*) pairs of horizontal and verticle bars in pressure pulse trace #1 depict the range of sampling variability for a typical heart due to the approximately 10 msec required to effectively freeze-clamp the organ to 0°C. The arrow in pressure pulse 3 is an example indicating the precise point of inflection of the pressure signal representing the moment during the contraction cycle at which the heart was frozen. The freezing point was verified by monitoring the pressure signal with a memory oscilloscope. Hearts developed an average peak systolic pressure of 95 mm Hg.

The rectangles on pressure pulse 1 of Figure 1 demonstrate the effect upon sampling resolution of the 10 msec variability inherent in the freeze-clamping methodology. With
the high degree of sensitivity provided by the electronic control mechanisms of the apparatus, coupled with the manner of segmentation of the cardiac cycle, the inter-sample variability was effectively minimized. The abrupt change shown by the arrow in pressure pulse 3 of Figure 1 indicates the precise point at which the heart is arrested by freeze clamping. As indicated in the Methods, this point was confirmed with a memory oscilloscope.

The cardiac cycle was segmented into phases by 3 different schemes (A-C) as illustrated on the 3 pressure pulses of Figure 2. Since cAMP levels have been reported to increase in the first 10-30% of systole (10,141,144), the methods of cardiac cycle segmentation shown in the pressure pulses labelled as A and B in Figure 2 were utilized to subdivide the contraction and relaxation components of the cycle. In pressure pulse A, the phase 1A subdivision encompassed diastole to a boundary representing approximately 25% of peak pressure development (PPD). Phase 2A continues from the upper boundary of phase 1A to a cut-off point representing PPD. Phase 3A represents the period of the cardiac cycle from PPD to diastole, a phase felt to correspond to relaxation related events in the cycle. In pressure tracing B of Figure 2, the cardiac cycle was subdivided into 4 phases, with the 2 phases in systole representing the period from diastole to 50% PPD (phase 1B) and 51% PPD to PPD (phase 2B) and the 2 phases during relaxation delineating the periods from PPD to a period
corresponding to a 50% reduction in pressure (phase 3B) and a final phase (4B) occurring from the lower boundary of phase 3B to diastole. Scheme C in Figure 2 segments the cardiac cycle into 2 phases, diastole to PPD (phase 1C) and PPD to diastole (phase 2C).

FIGURE 2. Cardiac cycle segmentation schemes of perfused rat hearts. The cardiac cycle was segmented by 3 different schemes (A, B, C), into 2 to 4 phases per cycle. The limits of each phase are delineated by the triangles adjacent to each pressure pulse and defined in Results. Individual phases were numbered in a manner to uniquely identify a particular phase in a given mode of cardiac cycle segmentation (ie., 1A = phase 1, segmentation methodology A). The position of the phase label on the pressure pulse tracing represents the mean time point in the phase at which the hearts were frozen.
The myocardial levels of cAMP as well as the percent activation of cAMP-dependent protein kinase (PKA) and glycogen phosphorylase (plase) for the different phases of segmentation schemes (A-C) are shown in Table I.

<table>
<thead>
<tr>
<th>Segment(n)</th>
<th>cAMP</th>
<th>PKA</th>
<th>Plase (G-1-P)</th>
<th>Plase (G)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A (5)</td>
<td>4.00 ± 1.0</td>
<td>14.4 ± 1.2</td>
<td>11.8 ± 3.1</td>
<td>27.1 ± 2.8</td>
</tr>
<tr>
<td>2A (5)</td>
<td>3.92 ± 1.2</td>
<td>12.3 ± 1.2</td>
<td>12.3 ± 3.0</td>
<td>20.8 ± 2.9</td>
</tr>
<tr>
<td>3A (5)</td>
<td>2.63 ± 0.6</td>
<td>13.4 ± 1.6</td>
<td>12.9 ± 3.7</td>
<td>26.6 ± 3.7</td>
</tr>
<tr>
<td>1B (3)</td>
<td>4.15 ± 1.2</td>
<td>15.2 ± 1.3</td>
<td>12.1 ± 1.8</td>
<td>26.5 ± 5.2</td>
</tr>
<tr>
<td>2B (3)</td>
<td>2.58 ± 0.5</td>
<td>13.0 ± 1.1</td>
<td>8.73 ± 2.1</td>
<td>20.0 ± 4.1</td>
</tr>
<tr>
<td>3B (3)</td>
<td>2.49 ± 0.7</td>
<td>13.8 ± 3.3</td>
<td>11.5 ± 2.1</td>
<td>24.5 ± 7.0</td>
</tr>
<tr>
<td>4B (3)</td>
<td>3.05 ± 1.2</td>
<td>13.6 ± 1.0</td>
<td>12.6 ± 2.5</td>
<td>28.1 ± 2.8</td>
</tr>
<tr>
<td>1C (8)</td>
<td>3.78 ± 0.8</td>
<td>14.3 ± 0.9</td>
<td>10.9 ± 1.1</td>
<td>23.5 ± 2.9</td>
</tr>
<tr>
<td>2C (8)</td>
<td>2.77 ± 0.7</td>
<td>13.1 ± 1.3</td>
<td>12.2 ± 1.6</td>
<td>26.5 ± 3.1</td>
</tr>
</tbody>
</table>

Table 1. The content of cAMP and the percent activation of cAMP dependent protein kinase and glycogen phosphorylase during various phases of the cardiac cycle. The cAMP, cAMP-dependent protein kinase (PKA) and glycogen phosphorylase (plase) values represent the mean ± 1 SE for the number of hearts given in each phase. Cyclic AMP is expressed in pmol/mg protein. PKA percent activation is expressed as the ratio of activity -cAMP/+cAMP x 100. The percent activation of glycogen phosphorylase both in the direction of glucose-1-phosphate (plase, G-1-P) and glycogen (plase, G) formation is expressed as the ratio of activity -AMP/+AMP x 100. See Methods for further details.
In segmentation scheme A, cAMP levels ranged from 2.63 to 4.00 pmol/mg protein (1A-3A) and did not vary significantly during the cardiac cycle. The percent activation of PKA ranged from 12.3 to 14.4 and did not vary significantly over phases 1A to 3A. The percent activation of glycogen phosphorylase, determined in the direction of glucose-1-phosphate or glycogen formation ranged from 11.8 to 12.9 or 20.8 to 27.1, respectively, over phases 1A to 3A and did not vary significantly over the cardiac cycle.

In segmentation scheme B, cAMP levels ranged from 2.49 to 4.15 pmol/mg protein (1B-4B) and did not vary significantly over the cardiac cycle. PKA percent activation ranged from 13.0 to 15.2, with no significant differences over phases 1B to 4B. Glycogen phosphorylase percent activation as determined by the formation of glucose-1-phosphate or glycogen ranged from 8.7 to 12.6 or 20.0 to 28.1, respectively. There was no significant difference between phases for the activation of phosphorylase as determined by the formation of either product. In segmentation scheme C, cAMP levels ranged between 2.77 and 3.78 pmol/mg protein with no significant differences between phases. PKA percent activation ranged from 13.1 to 14.3 and did not significantly vary between phases. Glycogen phosphorylase percent activation, in terms of glucose-1-phosphate formation, ranged from 10.9 to 12.2, and from 23.5 to 26.5 in terms of the glycogen formation assay method. Neither
assay method demonstrated significant differences between phases of the cardiac cycle. Total PKA activity for all hearts ranged between 4.2 to 5.0 mmol phosphate/min/mg supernatant protein and did not vary in any phase for segmentation schemes A-C. Total glycogen phosphorylase activity also did not vary for any phase of these segmentation schemes and ranged between 6.2 to 7.8 mmol phosphate/min/mg supernatant protein for the formation of glycogen.

DISCUSSION

Analysis of a single cardiac cycle in the perfused contracting mammalian heart presents a fundamental difficulty. The short duration (250 msec in rat) of a single cycle has made it extremely difficult to accurately isolate a particular point of the cycle. In the amphibian heart the longer cycle duration (1 cycle/sec) has permitted a greater segmentation of systole and diastole. Additionally, the relative insensitivity to temperature of amphibian myocardium has greatly facilitated experimental manipulation. The amphibian tolerance to lower temperatures (10°C), coupled with the concomitant increase in the cardiac contraction cycle duration (2-3 sec/cycle), presents a larger sampling window and permits a more precise point to point resolution of the cycle. In the mammalian myocardium previous reports have described various metabolic changes from systole to diastole in the perfused heart (134,141). However, in these reported stud-
ies the methods of perfusion were significantly altered from the routine conditions employed in aortic retrograde perfusion techniques. One modified perfusion protocol, where an oscillation in cyclic AMP was observed, involved the use of low concentrations of isoproterenol and insulin added to a high Ca\(^{2+}\) perfusion medium delivered at an elevated perfusion pressure to stress the heart (141). This modified protocol could have produced an alteration in inotropic state, which may have compromised metabolic description of the cardiac cycle. In another study where an oscillation in phosphorylase was observed (134), the temperature of the perfused rat myocardium was lowered to 32°C. The sinoatrial node was destroyed by crushing, lidocaine was topically administered and Ca\(^{2+}\) was replaced in the perfusion media with Sr\(^{2+}\), all for the purpose of extending the length of the cardiac cycle from its normal duration of 250-300 msec to approximately 1 second in duration. In this instance, the marked variation from standard physiological conditions to produce an elongated contraction cycle must be considered in the interpretation of the physiological significance of the findings. Oscillations of cAMP, cAMP-dependent protein kinase, and glycogen phosphorylase were observed during the cardiac cycle in an anesthetized open-chest canine preparation but were not seen upon propanolol administration (78). These results suggested that the oscillations were dependent on beat-to-beat endogenous neuronal catecholamine release.
By maintaining perfusion conditions routinely used in working heart preparations it is believed the results presented herein are physiologically representative of the heart in vivo, particularly when catecholamine stimulation is absent. While no significant difference was noted in cAMP levels between the various phases of the cardiac cycle, higher values only tended to occur in the earlier phases of pressure development. These findings are in agreement with previous reports (24,78) indicating that cAMP does not vary during the cardiac cycle in mammalian preparations either free of catecholamine stimulation or in the presence of propanolol, a β-adrenergic antagonist. However, the present findings do not confirm the oscillations in cAMP reported for amphibian cardiac muscle (10,144). This disagreement could be due to species and/or preparation differences. The significance of cAMP variation during the cardiac cycle was examined in light of two distal biochemical events in the heart known to be influenced by the cyclic nucleotide, such as cAMP-dependent protein kinase and glycogen phosphorylase. Yet, no significant differences were noted in these cAMP-dependent events in the heart regardless of the method of cardiac cycle segmentation employed. These observations do not deny the possibility that cAMP may vary in a manner not detectable by techniques employed in this study. However, variation in these distal events, known to be influenced by cAMP modulation, most likely would have been observed
if the variation in cAMP levels were significant.

Previously reported changes in certain metabolites, such as creatine phosphate and ATP (38, 144) could be implicated, on a single cycle basis, to be changing as a direct result of biochemical events during a single cycle. When examined in view of the reaction kinetics of enzymes such as creatine kinase, with its rate of activity being sufficiently rapid to buffer any single cycle deficits in ATP, and actomyosin associated ATPase activity corresponding to the millisecond rate of cross-bridge turnover, it can be appreciated that certain metabolic events probably occur in a cyclical manner during a single cardiac cycle (7, 93). Additionally, in consideration of cellular energetics, it has been reported that the slow transport of NADH to the mitochondria demonstrates the limitations of glycolysis in meeting energy demands in the heart (7), whereas pyruvate perfused hearts have shown the importance of high energy phosphates in beat-to-beat metabolic events (141). It is also unlikely that a second messenger such as cAMP could produce the multiple, sequential changes in activities of the cascade of enzymes implicated in the coordination of events during a single mammalian cardiac cycle, given the current understanding of enzyme response to hormone induced changes in cAMP levels. It has been shown in the rat heart that cAMP response to epinephrine occurs within 5 secs of hormone administration, and while cAMP-dependent protein kinase activation follows a similar
time course, glycogen phosphorylase activation demonstrates a lag of 5-15 secs (22). While such changes are extremely rapid, events associated with the mammalian cardiac contraction cycle would require responses on the order of milliseconds (such as actomyosin associate ATPase) rather than seconds. Even considering the reported compartmentalization of cAMP (91,133) and protein kinase (20) within the myocyte, the various proposed translocations required may be too slow to provide a modulatory effect during a single cardiac cycle. Furthermore, it has been previously reported that no change in cAMP was observed between systole and diastole in isolated guinea pig papillary muscle (29). While the concept of localized compartments or pools of cAMP and protein kinase would present an attractive mechanism for cardiac cycle regulation, the concept has not been without drawbacks. Given the current appreciation of ventricular myocyte ultrastructure (7), the definitive demonstration of intracellular compartments has been difficult, and while associations may be made to the cytosolic or membrane associated fractions, little evidence is available to allow specific localization to areas such as sarcolemma, sarcoplasmic reticulum, mitochondria, etc. (20,52). Traditionally the role of a second messenger such as cAMP has been to produce slower, longer term responses rather than those associated with the rapid on and off steps seen in events such as the cyclic regulation of the ATPase associated
with the actomyosin complex. Consequently, oscillation in cyclic nucleotides (cAMP) during a single cardiac cycle would appear to represent a localized metabolic event or a minor metabolic variant rather than a significant biochemical modulator.

In summary, it was found that cAMP levels in the myocardium remained unchanged during the cardiac cycle in the contracting heart developing pressure in the absence of added catecholamines. Myocardial cyclic AMP-dependent protein kinase and glycogen phosphorylase activities also remain constant during a single cycle.
Acknowledgement

The assistance of Dr. Richard Fenton regarding the theory and implementation of the automated clamping device was greatly appreciated.

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Preliminary report of this work has been presented as an abstract (FASEB J. 2:A984, 1988).
CHAPTER III

ENDOGENOUS PROTEIN KINASE ACTIVITIES OF CARDIAC SARCOLEMMA AS DETERMINED BY SYNTHETIC PEPTIDES

ABSTRACT

Cardiac sarcolemma (SL) display ion channel, receptor and transport functions that are presumably influenced by membrane protein phosphorylations. Since protein kinase activity is important for protein phosphorylation, a highly purified canine SL preparation was examined for associated protein kinase activities utilizing synthetic peptide substrates. Cyclic AMP-dependent protein kinase activity of the membranes was $1606 \pm 193$ nmol $^{32}\text{P}/\text{min}/\mu\text{g}$ SL using KEMPTIDE as the substrate. \textit{IP$_2$O}, a site specific inhibitor of cAMP-dependent protein kinase blocked this activity. Calcium/calmodulin-dependent protein kinase activity, determined with a specific calcium/calmodulin-dependent protein kinase substrate, was $353 \pm 59$ nmol $^{32}\text{P}/\text{min}/\mu\text{g}$ SL and dependent on the presence of calmodulin. Cyclic GMP dependent protein kinase activity, measured using an H2B heptapeptide, was $35.7 \pm 6.9$ nmol $^{32}\text{P}/\text{min}/\mu\text{g}$ SL and only observed in the presence of exogenously added cGMP. Calcium/phospholipid dependent protein kinase activity, measured using a synthetic peptide analogous to a segment of the EGF receptor, was $13.2 \pm 2.4$ nmol $^{32}\text{P}/\text{min}/\mu\text{g}$ SL and EGTA sensitive. In vitro SL phosphory-
lation, performed in the absence and presence of IP$_{20}$, support the results observed with the synthetic substrates. While calcium/calmodulin-dependent protein kinase, cGMP-dependent protein kinase and calcium/phospholipid-dependent protein kinase are all associated with cardiac SL, cAMP-dependent protein kinase appears the predominant sarcolemmal kinase.
Introduction

Protein phosphorylation reactions, particularly those stimulated by the intracellular mediators, cAMP, cGMP and calcium are recognized as physiologic effectors mediating the actions of various regulatory processes in the mammalian heart. In the myocardium, protein phosphorylation has been associated with changes in ion channel activity (137), alterations in myofibrillar interactions (40), modulation of receptor sensitivity (33) and the regulation of enzyme activity (123). Although it is evident that regulatory processes utilizing protein phosphorylation as an effector are most often expressed as the integration of several signals converging upon one or more end points; i.e. a single channel type or a number of different contractile proteins, the study of protein phosphorylation has provided the ability to focus on individual steps in an often complex series of events.

The critical role of protein kinases in phosphorylation reactions has been described from many viewpoints. Since protein phosphorylation has been shown to be a critical component of transmembranal signaling (106), the actions of protein kinases at the level of the cardiac sarcolemma as well as their sarcolemmal and cytoplasmic evoked responses have become an intense area of investigation.

Cyclic AMP-dependent protein kinase, perhaps the most extensively examined kinase in the myocardium, has
been shown to have a variety of endogenous protein substrates. The functions of this kinase in the mammalian heart include phosphorylation of proposed sarcolemmal Ca\textsuperscript{2+} channel associated protein (114), and sarcoplasmic reticulum associated protein, phospholamban (89), myofibrillar regulatory proteins, troponin I and C-protein (40) and the covalent modification of intracellular enzymes (79). Another cyclic nucleotide-dependent protein kinase, cyclic GMP-dependent protein kinase has been reported to phosphorylate phospholamban and to stimulate sarcoplasmic reticulum Ca\textsuperscript{2+} uptake (108). Additionally, two calcium-dependent protein kinases have been examined in the myocardium. Calcium/calmodulin-dependent protein kinase is believed to phosphorylate a sarcolemmal protein thought to be associated with the slow inward Ca\textsuperscript{2+} channel, as well as the sarcoplasmic reticular protein, phospholamban. Both of these actions are felt to potentiate the role of cAMP-dependent protein kinase in the phosphorylation of these same proteins (123). Calcium/phospholipid-dependent protein kinase has been shown to phosphorylate in an additive manner and in conjunction with cAMP-dependent protein kinase and calcium/calmodulin-dependent protein kinase (63). Although the function of this kinase in the myocardium is uncertain, with its phosphorylation of a Ca\textsuperscript{2+} channel associated protein in the SL, calcium/phospholipid-dependent protein kinase may play an important role in cell-surface signal regulated cardiac function (137,148).
While much has been done to describe the role of particular SL-associated kinases, as well as their specific substrates, little has been offered to quantify the kinase activity in a specific manner, given the fact that a single endogenous substrate may be the target of several kinases. Many protein kinases are thought to recognize a site of action by virtue of the amino acid sequence surrounding the target residue(s) (37). Synthetic peptide substrates, with sequences designed to correspond to the sites phosphorylated in the intact protein, have been developed to specifically quantify a particular kinase, while minimizing or eliminating interference by other kinases which may also be present.

In this study four such synthetic substrates were used:

1. Kemptide, a substrate designed for specific recognition by cAMP-dependent protein kinase (74),
2. a peptide corresponding to the amino terminal residues of skeletal muscle glycogen synthase for determining calcium/calmodulin-dependent protein kinase activity (103),
3. a 23 residue peptide corresponding to a cytoplasmic segment of the EGF receptor for determining calcium/phospholipid-dependent protein kinase activity (62) and
4. a heptapeptide analogous to a segment of histone IIb for the assay of cGMP-dependent protein kinase activity (43). With these peptides we assessed the activity of various endogenous protein kinase activities in a highly enriched canine
sarcolemmal preparation. In addition we assessed the native activity of these kinases to phosphorylate endogenous protein substrates in this SL preparation, and found that the same protein substrate was phosphorylated by more than one protein kinase. In fact there was a strong correlation between the most active kinases, as determined by synthetic substrates, and the greatest degree of phosphorylation of endogenous SL protein substrates.

METHODS

Dogs used in this study were maintained and used in accordance with recommendations in the Guide for the Care and Use of Laboratory Animal, prepared by the Institute of Laboratory Animal Resources, National Research Council, U.S. Department of Health and Human Services, National Institutes of Health Publication No. 85-23, rev. 1985 and the Guidelines of the Animal Care Advisory Committee of the University of Massachusetts Medical School.

Isolation of Canine Cardiac Sarcolemma. Highly enriched sarcolemmal vesicles were prepared from canine left ventricle after a modification of the method of Jones (68). In brief, 150-200 g of left ventricle wall was dissected free of fat and surface arteries and minced in a Waring meat grinder. The minced tissue was separated into 30 g aliquots, placed into 250 ml polycarbonate centrifuge bottles (Sorvall) with 120 ml of a solution containing 10 mM Histidine and 0.75 M NaCl (Solution I) and homogenized
for 5 sec using a Polytron (Brinkman Instr.) PT20 set a half maximal speed. Following a 20 min centrifugation (4°C) at 14,000 x g in a Sorvall RC-5 centrifuge using a GSA rotor, the supernatant was discarded, the pellet was resuspended in 120 ml of fresh solution I and the above homogenization was repeated. After centrifuging as described above, the supernatant was again discarded and the pellet was resuspended in 120 ml of a solution containing 5 mM Histidine and 10 mM NaHCO₃ (Solution II), with the above homogenization and centrifugation repeated. Again, the supernatant was discarded and the pellet was resuspended in 100 ml of solution II. Each suspension was then homogenized for 3, 30 sec periods. Following a 20 min centrifugation at 14,000 x g, the pellets were discarded while the supernatants were pooled and aliquoted into 50 ml polyethylene tubes (Sorvall) and centrifuged for 30 min at 44,000 x g in an SS-34 rotor. The pellets were suspended in 30 ml chilled deionized water and then combined with an equal volume of a solution containing 2 M sucrose, 300 mM NaCl, 50 mM tetrasodium pyrophosphate and 100 mM Tris (pH 7.1). Ten ml aliquots were placed in Ti 70 ultracentrifuge tubes and gently covered with 7 ml of a solution containing 0.6 M sucrose, 300 mM NaCl, 50 mM tetrasodium pyrophosphate and 100 mM Tris (pH 7.1). The remainder of the tube (approximately 7 ml) was carefully topped off with a solution containing 0.25 M sucrose and 10 mM Histidine. The sealed tubes were centrifuged in a
Beckman L8-70M ultracentrifuge at 360,000 x g (4°C) for 54 min using a Ti 70 rotor. After centrifugation, the cloudy band (containing the most tightly sealed vesicles) appearing at the 0.25/0.6 M sucrose interface was collected by syringe, mixed with an equal volume of chilled deionized water and centrifuged at 150,000 x g in a Ti 70 rotor for 30 min (2°C). The pellets were resuspended in the buffer containing 0.25 M sucrose and 10 mM histidine and stored in 0.25 ml aliquots at -70°C for later use. The protein yield was approximately 15-20 mg sarcolemmal protein per 200 g ventricle as determined by Lowry protein assay (17) with bovine serum albumin as a standard.

All dissection and tissue processing were performed in a temperature controlled room (4°C) with all solutions kept on ice and all tissue homogenization performed in an ice/water slurry.

**Assay of Protein Kinases.** Utilizing kinase specific synthetic substrates, in the presence of [γ-32P]ATP (0.5 μC/μl), the phosphorylated substrates were isolated using a phosphocellulose/phosphoric acid assay system (113). For each assay, sarcolemmal samples were washed twice (50,000 x g, 15 min) and resuspended in 10 mM histidine to a final protein concentration of 1 mg/ml. All reactions were carried out in the presence of 25 mM HEPES (pH 7.2), 10 mM MgCl₂, 1 mM ATP, and 1 mg/ml bovine serum albumin. All assays, with the exception of a portion of the cAMP-dependent protein kinase (+cAMP), were conducted in the
presence of 1 μg/ml IP$_{20}$, a protein kinase A inhibitor (17). All assays, with the exception of the calcium/phospholipid-dependent protein kinase (+Ca$^{2+}$ portion) and calcium/calmodulin-dependent protein kinase (+Ca$^{2+}$ portion), were conducted in the presence of 5 mM EGTA. Trifluoperizine, an inhibitor of calcium/calmodulin-dependent protein kinase activity (27), was used in the assay for control activity of calcium/calmodulin-dependent protein kinase as well as for both steps (±Ca$^{2+}$) of the assay for calcium/phospholipid-dependent protein kinase.

In general, a 25 μl aliquot (1 μg/μl SL protein) of sarcolemma was added to a reaction vessel containing either the substrate peptide (100 μM) and the necessary cofactors or the peptide and agents or conditions to inhibit phosphorylation, as follows. Cyclic AMP-dependent protein kinase was assayed using Kemptide (1 μg/μl) in the presence and absence of 5 μM CAMP (12) and 1 μg/μl IP$_{20}$ (17). Ca$^{2+}$/phospholipid dependent-protein kinase was assayed using a synthetic peptide (62) modeled from the epidermal growth factor receptor in the absence and presence of 100 μM CaCl$_2$. Ca$^{2+}$/calmodulin dependent-protein kinase was assayed using a synthetic peptide substrate (103) modeled from a segment of skeletal muscle glycogen synthase in the absence and presence of 100 μM CaCl$_2$. Cyclic GMP-dependent protein kinase (PKG) was assayed using a synthetic substrate (43) modeled from a
segment of histone IIB (calf thymus).

For each kinase assay, following a 3 min incubation (30°C), a 50 µl aliquot was removed from the reaction vessel, spotted on a phosphocellulose disc (Whatman P-81) and immediately dropped into a circulating bath containing chilled 75 mM phosphoric acid (10-15 ml/disc) for 10 minutes. In some cases the incubation periods were varied to determine the linearity of the reactions with time. After 2 changes of the acid wash, and a final 5 min wash in 50% ethanol the discs were dried and the radioactivity was determined by liquid scintillation spectroscopy (Packard Tri-Carb 4530, Ecoscint diluent).

**In Vitro Endogenous Phosphorylation of Sarcolemmal Vesicles.** Washed vesicles, reconstituted as described for the kinase assays, were freeze-thawed in an acetone/dry ice mixture 15 times to ensure exposure of the cytoplasmic face prior to phosphorylation. An aliquot containing 100 µg SL protein was incubated in a 30°C water bath under conditions identical to those listed for the assay of the various protein kinases with the following exceptions. Synthetic peptides were not used in the reaction mix. Reactions were terminated with the addition of chilled trichloroacetic acid, to a final concentration of 5%, and placed on ice. The trichloroacetic acid precipitation step was used to separate the $^{32}$P bound to the substrate proteins from the unbound [$\gamma^{32}$P]ATP. Samples were centrifuged for 30 min (3,500 x g, 4°C) in an HS-4 (Sorvall)
rotor with the precipitates reconstituted in an electrophoresis buffer to yield a solution containing 1 mg/ml protein, 10% glycerol, 2% SDS, 0.6% Tris, 5% β-mercaptoethanol and 0.01% bromophenol blue and placed in a 95°C water bath for 60 sec.

Gel Electrophoresis and Autoradiography. Proteins were separated using a discontinuous SDS/PAGE system with a 12% polyacrylamide (2.8% cross-linker) separating gel and a 5% stacking gel after a modification of the method of Laemmli (82). Approximately 65 μg of the phosphorylated sarcolemmal protein was added to each gel lane and after a 20 min period at 20 mA (constant current) the current was increased to 35 mA and the gel was run until the tracking dye flowed from the bottom of gel (3.5-4 hours). Gels were fixed and stained in a 25% methanol/10% acetic acid solution containing 0.5% Coomassie Blue (G250), destained in a 5% glycerol/5% acetic acid solution and vacuum dried onto sheets of Whatman #3 filter paper or dialysis membrane (BioRad). The degree of protein phosphorylation (³²P incorporation) was determined using autoradiography (Kodak, XAR-5 film, Dupont, Lightning plus enhancing screen). Exposure periods of 2-4 days were utilized to optimize for film sensitivity. Both stained proteins and autoradiographic images were quantified using a computerized video densitometer.

Quantification of ³²P incorporation into endogenous substrates. Endogenous protein substrates were quantified on
SDS/polyacrylamide gels prior to drying on filter paper or dried on transparent dialysis membrane prior to densitometry. Bovine serum albumin and glycogen phosphorylase (rabbit skeletal muscle) were utilized as standards. The amount of $^{32}$P incorporated into each protein substrate was determined by comparison to standards containing known amounts of $^{32}$P placed in each film cassette along with the gel.

Materials. [$\gamma^{32}$P]ATP was purchased from Amersham Corporation. Kemptide, trifluoperazine, EGTA and calmodulin were purchased from Sigma Corporation. ATP, cAMP and cGMP were obtained from Boehringer-Mannheim Corporation. X-ray film and β-mercaptoethanol were obtained from Eastman-Kodak. All electrophoresis reagents were obtained from National Diagnostics Corporation. All other chemicals were of reagent grade or better and were obtained from commercial suppliers. The synthetic peptide substrates for the assay of calcium/phospholipid-dependent protein kinase, cGMP-dependent protein kinase and calcium/calmodulin-dependent protein kinase were kindly provided by Dr. L. G. Heasley, Dept. of Biochemistry, National Jewish Center for Immunology and Respiratory Medicine, Denver, Colorado.
RESULTS

ACTIVITY OF ENDOGENOUS CARDIAC SARCOLEMMAL PROTEIN KINASES. The time course for $^{32}$P incorporation (phosphorylation) into the various synthetic peptides was examined to determine an optimal period of incubation. Peptides are incubated for varying time periods in the presence of 25 $\mu$g sarcolemmal protein (Figure 3), under the conditions described in Methods.

![Graph showing time course of $^{32}$P incorporation into kinase specific synthetic peptide substrates.](image)

Figure 3. Time course of $^{32}$P incorporation into kinase specific synthetic peptide substrates. Freeze-thawed canine sarcolemmal microvesicles (25 $\mu$g) were incubated for the times indicated with synthetic substrates (100 $\mu$M) as described in Methods. Counts per minute (cpm)/$\mu$g sarcolemmal protein were determined by liquid scintillation spectroscopy, with PKA and PKCM values expressed by the scale on the left ordinate and PKG and PKC values expressed by the scale on the right ordinate. Refer to the legend of Table I for kinase abbreviations. All values are those determined after subtraction of basal levels of kinase activity.
All peptides were used at a final concentration of 100 μM, a value previously reported as optimal (39,58,75,99). With the exception of the +cAMP portion of the cAMP-dependent protein kinase assay, the protein kinase inhibitor protein IP20 was used in all assay systems to eliminate interference due to endogenous cAMP-dependent protein kinase activity. When examined with regard to counts per minute (over control) per μg of sarcolemmal protein, all the measured kinases displayed a positive slope linearity up to 8 minutes into the incubation period. This indicated that the assay system, particularly over the 3-5 min time period used, is not substrate limited with regard to either the synthetic substrate or [γ-32P]ATP. While no difference in enzyme activity was seen in the freshly thawed versus the freeze thawed vesicles, for the sake of continuity, only freeze thawed vesicles were utilized in all phases of the study.

The endogenous sarcolemmal protein kinase activities (Table II) in the enriched vesicular preparation, were determined using the various kinase specific synthetic substrates (29,58,75,99), under conditions as outlined in Methods. These activities are thought to reflect protein kinase related events associated with the sarcolemma. All assays (except as noted) were performed with the cAMP-dependent protein kinase inhibitor protein IP20 present at 1 μg/ml to eliminate any endogenous cAMP-dependent protein kinase activity.
Cyclic AMP-dependent protein kinase: The activity of protein kinase A, assayed using the synthetic peptide, Kemptide, was 1606 nmol $^{32}$P/min/μg SL, the highest activity in the preparation. Even with an excess (10X) of cAMP-dependent protein kinase inhibitor protein (IP$_{20}$) present (17) in the assay system there was still an activity of approximately 0.090 nmol $^{32}$P/min/μg SL present, suggesting either a slight inefficiency in the inhibitory capacity of IP$_{20}$, or perhaps the presence of a small amount of non cAMP-dependent phosphorylation of the Kemptide.

Table II: Endogenous sarcolemmal kinase activities

<table>
<thead>
<tr>
<th>Kinase</th>
<th>Substrate</th>
<th>Activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKA</td>
<td>Kemptide</td>
<td>1606 ± 193</td>
</tr>
<tr>
<td>PKCM</td>
<td>SMGS</td>
<td>353 ± 59</td>
</tr>
<tr>
<td>PKC</td>
<td>EGF Receptor</td>
<td>13.2 ± 2.4</td>
</tr>
<tr>
<td>PKG</td>
<td>H2B Heptapept.</td>
<td>35.7 ± 6.1</td>
</tr>
</tbody>
</table>

Sarcolemmal vesicles were isolated from canine ventricle and incubated with the listed synthetic substrates as described in Methods. PKA: cAMP-dependent protein kinase, PKCM: Ca$^{2+}$/calmodulin-dependent protein kinase, PKC: Ca$^{2+}$/phospholipid-dependent protein kinase, PKG: cGMP-dependent protein kinase, SMGS: skeletal muscle glycogen synthase, EGF: epidermal growth factor, H2B: histone 2B heptapeptide. Values are the means ± SE for 4-6 different sarcolemmal preparations. * nmol$^{32}$P/min/μg sarcolemmal protein.
Calcium/calmodulin-dependent protein kinase: The activity of calcium/calmodulin-dependent protein kinase was assayed using a synthetic peptide modeled after the site of phosphorylation of myosin light chain (103). The activity of 353 nmol $^{32}P/min/\mu g$ SL was completely abolished by trifluoperazine at a concentration of 1.0 $\mu M$.

Calcium/phospholipid-dependent protein kinase: The activity of protein kinase C was assayed using a synthetic peptide modeled to the sequence of the EGF receptor, an endogenous substrate for calcium/phospholipid-dependent protein kinase (62). This activity was 13.2 nmol $^{32}P/min/\mu g$ SL and was completely eliminated with 10 mM EGTA. When the phorbol ester 12-o-tetradecanoylphorbol 13 acetate was used at a concentration 1.0 $\mu M$, the calcium/phospholipid-dependent protein kinase activity was seen to increase to approximately 50 nmol $^{32}P/min/\mu g$ SL (data not shown). However, 12-o-tetradecanoylphorbol 13 acetate stimulated activity may not reflect normal physiological conditions (56,148) and consequently was not used in the method of assay.

Cyclic GMP-dependent protein kinase: The activity of cGMP-dependent protein kinase was assayed with a synthetic peptide (43) corresponding to the site phosphorylated by this enzyme on the HIIb histone (calf thymus). The activity level of 35.7 nmol $^{32}P/min/\mu g$ SL was undetectable without the addition of cGMP (100 $\mu M$).
**ENDOGENOUS SARCOLEMmal SUBSTRATE PHOSPHORYLATION.** The native activity of 4 protein kinases with respect to endogenous protein substrates, was determined in the freeze-thawed membranes from canine cardiac muscle, prepared as described in Methods. Protein substrate phosphorylations, as determined by the $^{32}$P incorporation into several protein substrates (Figure 4), were examined under control and activating conditions (as detailed in Methods) for cAMP-dependent protein kinase, calcium/calmodulin-dependent protein kinase, calcium/phospholipid-dependent protein kinase and cGMP-dependent protein kinase.

Three distinct protein bands, with approximate molecular weights of 15 kD, 28 kD and 90 kD (Figure 4, arrows a,b,c; d,e; and f,g, respectively), were phosphorylated by endogenous kinase activity. The distinct phosphorylations of the 3 proteins (Figure 4, lane 8), seen when reaction conditions are optimized for cAMP-dependent protein kinase, are still evident when the reaction conditions are employed to inhibit cAMP-dependent protein kinase and favor other endogenous kinases. Calcium/calmodulin-dependent protein kinase (Figure 4, lane 4) phosphorylates these proteins, although to a lesser extent than that seen with cAMP-dependent protein kinase. Calcium/phospholipid-dependent protein kinase (Figure 4, lane 6) demonstrated a slight phosphorylation of the 15 kD band. No detectable phosphorylation of sarcolemmal sub-
trate(s) was observed when endogenous cGMP-dependent protein kinase activity was examined (Figure 4, lane 2).

Figure 4. A representative autoradiogram of 12% SDS/polyacrylamide gel of sarcolemmal proteins. Canine sarcolemmal vesicles were prepared and incubated as described in Methods. Each lane was loaded with 65 μg sarcolemmal protein. Apparent molecular weight in kilo-Daltons (kD) is indicated in the left margin. Lanes 1, 3, 5 and 7 are control. Lanes 2, 4, 6, 8 are PKG, PKCM, PKC and PKA, respectively. a, b, c; d, e; and f, g represent the endogenous protein substrates with apparent molecular weights of 15kD, 28kD and 90kD, respectively. See legend of Table I for kinase abbreviations. Arrows along right margin (from bottom to top) indicate apparent molecular weights of 15kD, 28kD and 90kD.
The quantification of $^{32}\text{P}$ incorporation by the endogenous protein substrates seen in Figure 4 was performed on 5 different SL preparations with the following results: **cAMP-dependent protein kinase:** Cyclic AMP-dependent protein kinase is the most active of the endogenous kinases quantified in the sarcolemmal preparation with the ability to transfer 2.7 and 29.4 nmol $^{32}\text{P}/\text{min}/\mu\text{g}$ SL protein to the 15 kD and 28 kD protein substrates, respectively, and 10.3 pmol $^{32}\text{P}/\text{min}/\mu\text{g}$ transferred to the 90 kD substrate. **Ca$^{2+}$/calmodulin-dependent protein kinase:** Calcium/calmodulin-dependent protein kinase transferred 0.29, 17.1 and 7.4 pmol $^{32}\text{P}/\text{min}/\mu\text{g}$ to the 15 kD, 28 kD and 90 kD protein substrates, respectively. **Ca$^{2+}$/phospholipid-dependent protein kinase:** Calcium/phospholipid-dependent protein kinase phosphorylated the 15 kD protein at the rate of 0.16 nmol $^{32}\text{P}/\text{min}/\mu\text{g}$. **Cyclic GMP-dependent protein kinase:** No endogenous substrate phosphorylation was detectable due to cGMP-dependent protein kinase activity using the method employed.

**DISCUSSION**

This study was undertaken to examine the endogenous protein kinase activity at the level of the sarcolemma, with two basic methods of assessment employed. Utilizing kinase specific synthetic peptides, the level of native activity was determined for cAMP-dependent protein kinase, Ca$^{2+}$/calmodulin-dependent protein kinase, Ca$^{2+}$/phospholip-
id-dependent protein kinase and cGMP-dependent protein kinase in a commonly used, highly purified, canine cardiac sarcolemmal preparation (68). These values in nmol $^{32}\text{p/min/\mu g SL}$ were 1606, 353, 13.2 and, 35.7, respectively. Additionally, using the same sarcolemmal preparation, the manner in which 3 endogenous protein substrates were phosphorylated by these endogenous kinases, was determined.

The synthetic substrates offer several distinct advantages over other methods of measuring kinase activity. Each substrate utilized has been shown to be extremely specific for a given kinase and has been shown to exhibit $K_m$ and $V_{\text{max}}$ values similar to native substrates (43,62,79,103).

The specificity of the synthetic substrate is most advantageous when compared to the methods of assaying protein kinase activity using one of the available histones (I, IIb, III etc.) as a substrate. While the affinity of a given enzyme for a histone substrate may be sufficient to provide an adequate signal, the lack of specificity of a particular histone for a given enzyme, coupled with the possible lack of purity of the histone, limits the confidence one may utilize in excluding other kinases as a source of measured enzyme activity. Furthermore, the synthetic substrates utilized exhibit a linear response (Fig. 1) over time, thereby permitting relatively short periods of incubation, as well as providing assur-
ance that neither the synthetic substrate nor the ATP present in the reaction is a limiting factor in measuring the activity of particular kinase. Additionally, the precise control of the reaction conditions and the use of the cAMP-dependent protein kinase inhibitor protein IP20, with the synthetic peptides, maximizes the ability to specifically quantify a particular kinase activity, while minimizing cAMP-dependent protein kinase as a compromising signal source in the measurement of non cAMP-dependent protein kinases (37).

The endogenous protein kinase activities associated with the sarcolemma, measured using the synthetic substrates, are somewhat different than values previously reported (81). These differences may in part be due to the normalization of the kinase activity to the amount of sarcolemmal protein present compared to other reports that normalize the activity to the total amount of protein or tissue utilized as starting material. When the measurements obtained in this study were adjusted for total starting material (tissue weight) used, the values were similar to other reported values (81), and discrepancies could be explained by differences in preparation and purification techniques employed.

The endogenous protein substrates phosphorylated by the various kinases (Fig 2) appear to be similar to proteins shown to be phosphorylated in the heart by several previous reports (63,86,107,137,143). In comparing the
kinase activities determined with the synthetic peptides, to the phosphorylation of the endogenous substrates, there is a strong correlation between the most active kinase when measured with a synthetic peptide, to the most active kinase with regard to the phosphorylation of endogenous protein substrates. An exception to this correlation was cGMP-dependent protein kinase. While showing a sarcolemmal level of activity equal of 35.7 nmol $^{32}$P/min/μg, cGMP-dependent protein kinase was not found to detectably phosphorylate any endogenous protein substrate in the preparation. This discrepancy is again, most likely due to the manner in which the sarcolemma was isolated. It is possible that the enzyme substrate was lost during isolation, that the enzyme properties were altered or that the substrate is not associated with the sarcolemma. Alternatively, phosphorylation of endogenous substrates could be masked by simultaneous operation of a phosphatase, although conditions employed would presumably minimize this effect. It is of interest that there is little evidence of endogenous substrates for this enzyme in the myocardium despite evidence for PKG activity in vitro (61).

Although calcium/phospholipid-dependent protein kinase has been shown to phosphorylate several cardiac sarcolemmal proteins, these reports were the result of exogenously added kinase (63,81). When coupled to the aforementioned differences in sarcolemmal preparations and potential species variation, the ability to describe
confidently an event of physiological significance may be comprised by the experimental manipulations. Also, the sarcolemmal preparation used in this study is felt to provide all the necessary lipid cofactors in the vesicle itself. Consequently the addition of exogenous lipids, as used in other studies (106), was unnecessary, if not inappropriate.

The identity of the endogenous proteins phosphorylated in the preparation is uncertain. It is believed that the 28 kD substrate is most likely the protein phospholamban, given its molecular weight and its tendency to dissociate into 6 kD and 12 kD subunits when boiled for longer periods (3-5 min) in a solution containing 2% SDS (106). Although this protein is thought to be a membrane component of the sarcoplasmic reticulum, it is probably present as a contaminant of this sarcolemmal preparation (71). While this preparation is believed to be extremely pure, it must be noted that angiotensin converting enzyme activity in these SL was seen to be greatly enhanced over values determined in homogenized myocardial samples (116,135). Since angiotensin converting enzyme activity in the heart resides primarily in the vascular endothelium, it is assumed that this sarcolemmal preparation contains considerable contamination from components of the heart other than the myocyte (71).

Although previous studies have found that an 88-92 kD protein is phosphorylated by calcium/phospholipid-
dependent protein kinase in sarcolemmal preparations (63,148), such phosphorylation was only observed in the presence of cAMP-dependent protein kinase or calcium/calmodulin-dependent protein kinase. The inability to detect this phosphorylation in the present investigation could again be the result of either exogenously added calcium/phospholipid-dependent protein kinase used in earlier studies or differences in the preparation of the sarcolemma.

The 15 kD protein that was phosphorylated by cAMP-dependent protein kinase, calcium/calmodulin-dependent protein kinase and calcium/phospholipid-dependent protein kinase appears similar to the sarcolemmal cytoplasmic face protein described by Presti et al. (106,107), in that it was only seen in membranes having been freeze thawed prior to phosphorylation to expose the cytoplasmic face of the sarcolemma. This protein is distinguished from phospholambam by virtue of the insensitivity of the protein to boiling in SDS prior to electrophoresis. While phospholambam can be broken down to its smaller molecular weight subunits, the 15 kD protein is stable and remains unchanged under similar treatment. Whether associated with a channel, receptor, transporter or some other component of the sarcolemma the function of this 15 kD protein remains to be established. However, it presumably plays some role in protein phosphorylation mediated events at the level of the sarcolemma (6,106,107).
In conclusion, cAMP-dependent protein kinase, calcium/calmodulin-dependent protein kinase, calcium/phospholipid-dependent protein kinase and cGMP-dependent protein kinase activities appear to be associated with a highly enriched canine cardiac sarcolemma preparation, with cAMP-dependent protein kinase clearly being the most active. Sarcolemmal phosphorylations by endogenous protein kinases support the results obtained by assay of the kinases using specific synthetic substrates, thereby demonstrating the value of these substrates as a method of quantifying membrane kinase activity. Additionally, endogenous sarcolemmal phosphorylation clearly demonstrates that a given sarcolemmal substrate may well be phosphorylated by several different kinases associated with the cardiac sarcolemma.
Preliminary report of this work has been presented

(FED. PROC. 46:5492, 1987)

This work was supported by

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ADENOSINE AND ACETYLCHOLINE REDUCE ISOPROTERENOL-INDUCED PROTEIN PHOSPHORYLATION OF RAT MYOCYTES

ABSTRACT

Adenosinergic and muscarinic agents have been shown to attenuate the catecholamine-induced augmentation of both protein phosphorylation and contractile state in perfused hearts. The attenuation by phenylisopropyladenosine (PIA) and carbamylcholine chloride (CARB) of the isoproterenol (ISO) induced incorporation of $^{32}$P into protein substrates was examined in isolated rat ventricular myocytes. $^{32}$P labeled myocytes exposed to ISO (0.1 μM, 30 sec) demonstrated up to an 8-fold increase of $^{32}$P incorporation into 3 protein substrates (155, 31, 6 kD). When myocytes were pre-incubated with either PIA or CARB for 60 sec prior to the ISO exposure, the ISO induced $^{32}$P incorporation (in the 31 kD and the 155 kD substrates) was attenuated. This reduction averaged 37% and 25%, respectively by 1 μM PIA and averaged only 23% and 11% for these substrates after pretreatment with 10 μM PIA. One μM CARB produced a 24% and 17% reduction in these same substrates while 10 μM CARB produced a 44% and 50% reduction. The effects of ISO were prevented by 10 μM propanolol. The inhibitory effects of PIA were antagonized by theophylline, sulfophenyltheophylline and DPCPX, whereas atropine
antagonized the inhibitory effects of CARB. Additionally, while PIA and CARB reduced the ISO-induced increase in cAMP-dependent protein kinase (PKA) activity by 48% and 41%, respectively, only CARB affected the ISO-elicited increase in cAMP levels, attenuating this response by 58%. The results indicate that PIA was less effective in attenuating ISO-induced \(^{32}\text{P}\) incorporation at higher concentrations than at lower concentrations. Moreover this compound was less potent than CARB at attenuating the effects of ISO. It is conceivable that this difference could be related to activation of stimulatory adenosine receptors (A\(_2\)) and/or a greater density of muscarinic receptors including multiple muscarinic pathways.
INTRODUCTION

The increase in contractile state of the myocardium produced by β-adrenergic agonists is thought to be due to a receptor coupled mechanism. This receptor system in the heart is believed to mediate its physiological actions via a second messenger system that ultimately results in an enhancement in specific protein phosphorylations (123). Adenosinergic and muscarinic agonists are known to attenuate β-adrenergic induced responses in the heart (22,87,88).

In the myocardium, the β₁ agonist, isoproterenol, causes an increase in the intracellular level of cyclic adenosine 3',5'-monophosphate (cAMP) by transmembrane coupling of the receptor via a GTP binding protein, Gₛ, to the enzyme adenylyl cyclase (115). This increase in cAMP in turn activates cAMP-dependent protein kinase (PKA), an enzyme known to effect the phosphorylation state of cytoplasmic and membrane associated proteins (25).

In the mammalian heart isoproterenol elicits an increase in contractile state that is associated with an enhanced phosphorylation of several proteins, as evidenced by protein substrate α²P incorporation (25,109). PKA is thought to catalyze the phosphorylation of a cardiac calcium channel associated protein, phospholamban (33,87), troponin I and the thick filament protein known as C protein (40). Additionally, it has been shown that ade-
nosinergic and muscarinic agonists attenuate the catecholamine-induced increase in contractile state of perfused hearts (36, 47, 87). Concomitant with this attenuation is a reduction in myocardial cAMP levels, a decrease in the level of protein phosphorylation of several substrates and reduced activation of PKA and glycogen phosphorylase (23, 27).

The antiadrenergic actions of both adenosinergic and cholinergic agonists in the heart are known to occur by means of cell-membrane receptors designated as adenosinergic (A₁) and cholinergic (M₂), respectively (58, 90). Both of these receptor types are thought to evoke a portion of their intracellular effects by means of a transmembrane GTP binding protein which couples the receptor to adenylyl cyclase (65). While there is evidence for the presence of both A₁ and A₂ adenosine receptors in the myocardium (116), the A₁ subtype appears to be predominant (58). Myocyte adenosine A₁ and A₂ receptors inhibit and stimulate respectively with regard to adenylyl cyclase and are presumably located on the extracellular surface (116). The extracellular muscarinic receptor, M₂, is inhibitory to adenylyl cyclase (90).

Investigations utilizing the perfused heart have benefited from the ability to correlate changes in pressure generation with metabolic changes, such as variations in enzyme activities and protein phosphorylation. However, the perfused heart has numerous cell types present
which emanate from muscle, nervous, vascular and connective tissue. Dispersed primary cultured ventricular myocytes, free of other cell types, improve the resolution and understanding of biochemical changes seen in the myocardium. These myocytes possess β-adrenergic, adenosine and muscarinic receptors that respond to isoproterenol, adenosine, and acetylcholine, as well as a functional adenylyl cyclase (12,92,116).

The purpose of this investigation was to examine the antiadrenergic effects of adenosine and acetylcholine on β-adrenergic receptor-mediated protein phosphorylation, PKA activation and cAMP formation using primary cultured rat ventricular myocytes.

METHODS

Rats used in this study were maintained and used in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals, prepared by the Institute of Laboratory Animal Resources, National Research Council, U.S. Department of Health and Human Services, National Institutes of Health Publication No. 85-23, rev. 1985 and the Guidelines of the Animal Care Advisory Committee of the University of Massachusetts Medical School.

MYOCYTE ISOLATION. Isolated, calcium tolerant ventricular myocytes were obtained from male Sprague-Dawley rats (275-350 g) by enzymatic disassociation using a combination of collagenase and hyaluronidase as previously de-
scribed (116). Isolated cells were plated on culture dishes that were preincubated for a minimum of 4 hours with media M199 (25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, HEPES, pH 7.4) supplemented with 5.6% heat inactivated fetal calf serum (FCS, Gibco). The final concentration of FCS after addition of the cells to the culture plates (approximately $10^6$ cells per 60 mm dish), was approximately 4%. Dishes were maintained from the pre-incubation phase through completion of the experiment in a humidified, quick recovery incubator (37°C) under constant gassing with a 5% CO$_2$-room air mixture. Preparations that did not yield a minimum of 60-70% rod shaped cells, as determined by microscopic inspection at final settling, were discarded.

**PROTEIN PHOSPHORYLATION.** Two hours after plating, the media and any accompanying debris were aspirated and replaced with fresh media containing 50 μCi per ml $^{32}$P as orthophosphate, with the myocytes at this point being approximately 90% rod-shaped. After 90 min, the media was replaced with fresh media of the same specific radioactivity of $^{32}$P, plus ISO, PIA, CARB, propanolol, etc. as indicated. Where utilized, adenosine deaminase (ADA), was introduced at 2 units/ml M199 during the 90 min period used for $^{32}$P loading of the cells. All experimental manipulations were carried out in the incubator to control temperature and the gaseous environment. The experimental periods were terminated by rapid aspiration of the media
followed by immediate flotation of the culture dish on liquid nitrogen. Cells were maintained at -75°C until harvested for analysis.

[γ-32P]ATP SPECIFIC RADIOACTIVITY. To ensure that specific radioactivity levels of ATP remained constant within preparations, [γ-32P]ATP specific activity levels in several preparations were examined over the various experimental manipulations utilizing a casein based assay system (14). Briefly, the cells were homogenized in 5% perchloric acid (PCA), the soluble extracts neutralized and the extract ATP levels determined fluorometrically. The neutralized extracts were then incubated in the presence of PKA, cAMP and casein. The incorporation of 32p into casein from [γ-32P]ATP was measured by PCA precipitation of the protein on filter paper. 32p-casein formation was linearly related to the specific radioactivity of the [γ-32P]ATP. The specific activity of the cell preparations did not vary from control when treated with ISO (1 μM) or ISO in combination with either PIA (1 μM) or CARB (10 μM).

CELL HARVESTING. In all cases, frozen cells were harvested over ice by scraping with a rubber policeman in an ice-chilled solution (0°C) using 2 x 650 μl washes per dish and placed into 1.5 ml centrifuge tubes (Eppendorf, polypropylene, Flex-Tube) on ice. For protein phosphorylation and immunotransfer, cells were harvested in a solution containing 100 mM KF and 5 mM EDTA, then centri-
4°C. The supernatant was discarded and the pellet was reconstituted in 300 μl of 1.0% sodium dodecyl sulfate (SDS). For cAMP determinations, cells were similarly harvested using a chilled solution of 65% ethanol, with the resultant suspension centrifuged as mentioned above. The supernatants were dried and reconstituted in a buffer containing 50 mM sodium acetate (pH 6.0) and 0.01% thimerosal and stored at -20°C until assayed. The pellet was resuspended in 1% SDS for protein determination. For cAMP dependent protein kinase assay, cells were harvested by the same manner as described above using a harvesting buffer containing 50 mM 3-(2,3)propanesulfonic acid (MOPS, pH 7.2), 0.1% Nonidet-P40 and 10 mM MgCl₂, and sonicated over ice (2 x 5 sec burst) to obtain a cellular homogenate. The resultant homogenate was assayed for PKA activity and total myocyte protein.

CYCLIC AMP DETERMINATION. The cAMP levels in the myocyte extracts reconstituted in the sodium acetate buffer were determined using an ¹²⁵I-cAMP RIA kit (Amersham). This assay employs a cAMP specific rabbit antibody, with the final separation being achieved by a second antibody (donkey) bound to a polymer, thereby permitting separation of the unbound labeled tracer by centrifugation. The cAMP values are reported in pmol/mg protein of the original ethanol pellet (total cell protein). In this laboratory, this manner of cAMP assay routinely provides recovery values in excess of 90% in similar cellular preparations.
values in excess of 90% in similar cellular preparations.

**PROTEIN KINASE ACTIVITY.** The level of cAMP-dependent protein kinase activity was determined by measuring $^{32}P$ incorporation into the synthetic peptide substrate, Kemp-tide, in a phosphocellulose/phosphoric acid assay system (113). The assay mixture contained 25 mM MOPS (pH 7.2), 10 mM MgCl$_2$, 1 mM ATP and 5 μCi $^{32}P$ as [γ-$^{32}P$]ATP per reaction vessel. The reaction was started by adding 10 μg of myocyte protein from the sonicated homogenate. The reaction was incubated for 5 min at 30°C, and then was terminated by spotting a 50 μl aliquot from the reaction vessel on a phosphocellulose disc (Whatman P-81). The disc was immediately placed into a chilled circulating bath of 75 mM phosphoric acid (10-15 ml/disc). After 2 changes of the acid wash and a 5 min rinse in 50% ethanol, the discs were dried and the radioactivity was determined by liquid scintillation spectroscopy. Protein kinase is expressed as the ratio of activity in the absence and presence of cAMP (2 μM). An increase in the activity ratio is assumed to reflect enzyme dissociation of the holoenzyme into its regulatory and active catalytic subunits. Kinase values are reported as percent activation and reflect the activity ratios (-cAMP/+cAMP) x 100.

**GEL ELECTROPHORESIS AND AUTORADIOGRAPHY.** The SDS solubilized myocyte particulate protein was combined with electrophoresis sample buffer to yield a solution containing 0.5 mg/ml protein, 10% glycerol, 2% SDS, 0.6% tris(hydrox-
0.01% bromophenol blue. Samples were then placed in a 95°C water bath for 60 seconds. Solubilized proteins were separated using a discontinuous SDS/PAGE system with a 12% polyacrylamide (2.8% cross-linker) separating gel and a 5% stacking gel, after the methods of Laemmli (82). Approximately 25 μg of protein were loaded to each gel lane and, after a 20 min period at 20 mAmps (constant current) the current was raised to 35 mAmps and the gel was run until the tracking dye cleared the bottom of the gel (3.5-4.0 hours). Gels were fixed and stained in a 0.5% Coomassie Blue (G250) solution containing 25% methanol/10% acetic acid, destained in a 5% glycerol/5% acetic acid solution and vacuum dried onto sheets of dialysis membrane (BioRad). The extent of 32P incorporation into the various protein substrates was determined using standard autoradiography (Kodak, XAR-5 film, Dupont, Lightning Plus enhancing screen) and was utilized as an index of the phosphorylation state(s) of the substrates. Exposure periods of 1-3 days were utilized to optimize the film's range of sensitivity. Both stained proteins and autoradiographic images were quantified using a computerized video densitometer (Imagemeasure software, IBM Personal Computer AT).

IMMUNOTRANSFER AND IMMUNOBLOTTING. At the conclusion of SDS/PAGE electrophoresis, gels of solubilized myocyte particulate protein were immediately subjected to immunotransfer and blotting utilizing commonly employed method-
ology (45). Briefly, the gel was soaked in a 20% methanol transfer buffer containing 50 mM Tris, 385 mM glycine and 0.01% SDS, for 30 min, then transferred overnight at 1000 mA constant current (Hoefer, Model TE 50) to nitrocellulose (0.22 μM pore, Micron Separation Inc.). Strips of nitrocellulose were blocked with 1% bovine serum albumin (BSA) and 0.05% polyoxyethylenesorbitan (Tween) in Tris buffered saline (TBS, pH, 7.5), rinsed in TBS and exposed to primary antibody (anti-rabbit skeletal TNI from mouse) for 60 min. Strips were then rinsed several times with 0.05% Tween in TBS and exposed to a secondary antibody (anti-mouse from goat) for 60 min. After several rinses in Tween/TBS, a color product was produced using a commercially available alkaline phosphatase kit (BioRad) and the product identification was confirmed by autoradiography.

**PROTEIN DETERMINATION.** Protein levels were determined with a commercially obtained assay (Pierce) utilizing a bicinchoninic acid-copper sulfate reaction with bovine serum albumin (BSA) used as a standard.

**MATERIALS.** All isotopes were purchased from Amersham Corporation. Kemptide, carbamyl choline chloride, propranolol, forskolin, atropine, albumin, and isoproterenol were obtained from Sigma Corporation. ATP, phenylisopropyladenosine (PIA) and cAMP were purchased from Boehringer-Mannheim Corporation. 5'-N-ethylcarboxamidoadenosine hydrate (NECA), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) and 8-sulfophenyltheophylline were obtained from Research
Biologicals, Inc. Collagenase and hyaluronidase were obtained from Worthington Biochemicals. Fetal calf serum and media 199 were purchased from Gibco Corporation. All electrophoresis reagents were purchased from National Diagnostics Corporation. All other chemicals used were of reagent or a superior grade and were obtained from commercial suppliers. Anti-cardiac TNI was kindly provided by Dr. K. D. Warber, Dept. of Pharmacology, University of Miami School of Medicine, Miami, Florida. The cAMP-dependent protein kinase inhibitor protein, IP$_{20}$, was kindly provided by Dr. L. G. Heasely, National Jewish Center for Immunology and Respiratory Medicine and the Department of Pharmacology, University of Colorado School of Medicine, Denver, Colorado.

STATISTICAL METHODS. All data are expressed as means ± one standard error of the mean. Statistical significance was determined using one way independent analysis of variance (122). A probability of less than 0.05 was accepted as indicating a statistically significant difference.

RESULTS

ISOLATED MYOCYTE VIABILITY. The myocytes utilized are easily obtained and physiologically viable. The cells shown in Figure 5a (25X photograph) are collagenase/hyaluronidase dispersed ventricular myocytes. These cells exclude trypan blue, are calcium tolerant (2.5 mM) and
reversibly contract in response to field stimulation (5 msec, 50 mV).

Figure 5. Isolated ventricular myocytes. Photograph of adult rat primary culture (2 hr) collagenase dispersed ventricular myocytes in medium M199 supplemented as described in Materials and Methods. Cells have been washed with fresh media after 90 min of incubation and are firmly adhered to the surface of the culture dish. (a) 25X. Cells are plated at approximately $10^6$/ml and demonstrate the typical rod-shaped morphology with the dark round spots representing damaged myocytes. (b) 300X. At higher magnification, the rectangular shape, sharp margins and the typical appearance of striations in the cells are evident.
In Figure 5b, these cells at higher magnification (300X) demonstrate the striations and abrupt cell margins characteristic of the morphology of the myocyte. While dead or dying cells may exclude trypan blue (121,140), the striations, distinct cell margins, and ability of the cells to reversibly contract when stimulated serve as strong indices of the viability of the cardiomyocytes obtained in this preparation. Preparations of less than 60-70% viable cells (as determined by morphology), or those that produced less than a 200% increase over control phosphorylation (as determined by $^{32}\text{P}$ incorporation into a 31 kD protein band) in response to a 30 sec exposure to 0.1 $\mu$M isoproterenol were typically discarded. Cells showed less than a 10% overall change in viability over 4 hours after initial plating on culture dishes.

**TIME COURSE OF PROTEIN $^{32}\text{P}$ INCORPORATION.** The isoproterenol (0.1 $\mu$M, ISO) stimulated incorporation of $^{32}\text{P}$ is time dependent, with the 31kD protein substrate demonstrating a 2 fold greater change in percentage incorporation over control than the 155kD substrate (Figure 6). The observed response to a 5 sec ISO (0.1 $\mu$M) exposure may be somewhat limited by the ability to ensure uniform mixing of the ISO. However, there was a significant increase in percent $^{32}\text{P}$ incorporation in both proteins (as expressed as a percent of control). The increase in $^{32}\text{P}$ incorporation into the 31kD and the 155kD proteins over periods of ISO exposure of 5-120 sec ranged from 29-904% and 125-365%
respectively, over control. Since the $^{32}$P incorporation into the 31kD protein was the greater in magnitude, this protein was selected as a reference, with the approximate half maximal response time of 30 sec as the time period for the majority of the experimental manipulations.

Figure 6. **Percent isoproterenol induced phosphorylation over control.** Time course of isoproterenol stimulated $^{32}$P incorporation into 31kD and 155kD protein substrates in ventricular myocytes expressed as a percent over untreated control. After a 90 min incubation in M199 media containing 50 μCi/ml $^{32}$P as described in Methods, adherent cells are exposed to isoproterenol for indicated lengths of time, with the media then immediately aspirated and the cultures flash frozen in liquid nitrogen. Values are the means ± SE for 4-10 different preparations. Note that ordinates are scaled differently. All values (5-120 sec) are significantly different from the appropriate control value and for the percent $^{32}$P incorporated by each protein at the same time point.
PROTEIN SEPARATION AND $^{32}$P INCORPORATION WITH ISOPROPYLNOL IN THE ABSENCE AND PRESENCE OF PHENYLISOPROPYLADENOSINE OR CARBACHOL. Myocytes readily demonstrated marked changes in $^{32}$P incorporation into various protein substrates. A polyacrylamide gel and its corresponding autoradiograph are shown in Figure 7 to demonstrate both the stained proteins being examined, as well as the extent of change in $^{32}$P incorporation seen as a result of a typical experiment. The 155kD protein is seen as a fine band appearing near the top of the gel (Figure 7A, top arrow), while the 31kD protein appears as a broad band (middle arrow). Both proteins show an ISO-induced (0.1 μM ISO, 30 sec) increase in $^{32}$P incorporation (Figure 7B), when lane a (control) is compared with lane b. The sample in lane c on the autoradiogram demonstrates the result of the myocytes receiving a 60 sec exposure to 1 μM phenylisopropyladenosine (PIA) immediately before being administered the same dose of ISO as used in lane b. The sample in lane d received a 60 sec pre-incubation to 10 μM carbachol (CARB) prior to the ISO exposure. It is evident that in the instances of pre-incubation with either PIA or CARB, the incorporation of $^{32}$P observed with ISO in the 31kD and 155kD protein substrates was less than that seen with ISO alone. It should be noted that near the bottom of the autoradiograph (Figure 7B, bottom arrow), yet not
clearly visible on the gel, is the trace of a protein of approximately 6kD. This protein appears to be the mono-

Figure 7. Gel electrophoresis and autoradiography. The 12% SDS/PAG gel (panel A, lanes 1-4) and resultant autoradiograph (panel B, lanes a-d) represent a typical protein separation and film exposure of the myocytes treated as described in the Methods. Lanes 1 and a - control. Lanes 2 and b - isoproterenol, 0.1 μM, 30 sec. Lanes 3 and c - Phenylisopropyladenosine, 1.0 μM, 60 sec, followed by isoproterenol, 0.10 μM, 30 sec. Lanes 4 and d - carbachol, 10 μM, 60 sec, followed by isoproterenol, 0.10 μM, 30 sec. Each lane was loaded with 20 μg myocyte (\(^{32}\)P loaded) protein obtained by harvesting the treated cells from frozen culture dishes for processing as detailed in Methods. The arrows represent the molecular weights in ascending order of 6kD, 31kD and 155kD.
meric form of phospholamban. This identification is based on the appearance of the protein in unboiled samples in its pentameric form of approximately 28kD, as well as the increase seen in $^{32}$P incorporation into this substrate in response to ISO. The ISO-induced $^{32}$P incorporation seen in the 6kD protein was attenuated by PIA and CARB in a manner similar to that observed in the 31kD and 155kD proteins.

**INHIBITION OF ISOPROTERENOL INDUCED $^{32}$P INCORPORATION BY PIA AND CARBACHOL.** Both PIA and CARB attenuated the ISO-induced incorporation of $^{32}$P into the 31kD and 155kD protein substrates. Samples preincubated for 60 sec with 1 or 10 $\mu$M or 10 $\mu$M CARB prior to exposure for 30 sec to 0.1 $\mu$M ISO showed an average inhibition of 37%, 23% and 47% respectively, for the 31kD substrate, and 25%, 11% and 35%, for the 155kD substrate, respectively (Figure 8). The 1 $\mu$M PIA concentration produced greater inhibition than the 10 $\mu$M, and 10 $\mu$M CARB was more effective than either dose of PIA in inhibiting the ISO-stimulated $^{32}$P incorporation seen in both the 31kD and the 155kD protein. When the cells were plated with adenosine deaminase present (2 units/ml M199) for the 90 min period of $^{32}$P loading of the cells prior to and during experimental manipulations, no difference in PIA inhibition of ISO-induced $^{32}$P incorporation was noted.
Figure 8. The inhibition of isoproterenol stimulated $^{32P}$ incorporation by phenylisopropylnadenosine, and carbachol. $^{32P}$ loaded myocytes are exposed to either phenylisopropylnadenosine (PIA, 1 $\mu$M or 10 $\mu$M) or carbachol (Cb, 10 $\mu$M) for 60 sec prior to a 30 sec exposure to isoproterenol (ISO, 0.1 $\mu$M). Each treatment represents the mean ± SE of 8-12 experiments. All values were significantly different when compared to the value with ISO alone. An asterisk denotes a significant difference compared to either concentration of PIA with ISO.

**RECEPTOR SPECIFICITY.** The specificity of the actions of ISO, PIA and CARB to influence protein $^{32P}$ incorporation is demonstrated in the autoradiogram (Figure 9) by using the antagonists, propanolol (10 $\mu$M), 8-sulfophenythio-
phylline (1 μM) or dipropylcyclopentylxanthine (DPCPX, 1 μM), and atropine (1 μM), respectively.

Figure 9. Specificity of isoproterenol, phenylisopropyladenosine and carbachol. The autoradiogram of a 12% polyacrylamide gel (SDS/PAG) loaded with 20 μg myocyte (32P loaded) protein per lane was obtained as described in Methods. The various agents were administered as follows. Exposure to 0.1 μM isoproterenol was for 30 sec. Prop: 60 sec prior to ISO. PIA: 60 sec prior to ISO. CARB: 60 sec prior to ISO. Theo: 60 sec prior to PIA. ATR: 60 sec prior to CARB. DPCPX: 60 sec prior to ISO. Lower and upper arrowheads indicate the 31kD and 155kD substrates, respectively. Lanes 1, 2 and, 15 are control (untreated myocytes). Lanes 3 to 8 and 11 to 14 were ISO. Lane 4 - Prop prior to ISO. Lanes 5 and 6 - PIA and CARB, respectively, prior to ISO exposure. Lane 7 - Theo, prior to PIA, which was prior to ISO. Lane 8 - ATR, prior to CARB, which was prior to ISO. Lanes 9 and 10 - Theo and ATR, respectively. Lane 11 - ISO. Lane 12 - DPCPX, which was prior to ISO. Lane 13 - DPCPX prior to PIA, which was prior to ISO. Lane 14 - PIA, which was prior to ISO. Prop = propranolol (10 μM), PIA = phenylisopropyladenosine (1.0 μM), CARB = carbachol (10 μM), THEO = 8-sulfophenyltheophylline (1.0 μM), ATR = atropine sulfate (1.0 μM), DPCPX = dipropylcyclopentylxanthine (0.10 μM).
The lanes marked as 1, 2 and 15 represent untreated (control) myocytes. Lanes 3 and 11 represent a 30 sec exposure to 0.1 μM ISO and demonstrate the 32P incorporation by the 31kD and 155kD proteins (lower and upper arrowheads, respectively, of Figure 5). 1 μM propanolol blocked the ISO-induced 32P incorporation into both proteins (lane 4). Lanes 7 and 8 demonstrate that the effects of PIA and CARB (lanes 5 and 6) are antagonized by 8-sulfophenyltheophylline (1 μM) and atropine (0.1 μM) respectively. 8-sulfophenyltheophylline (1 μM) and atropine (0.1 μM) alone produced no effect on phosphorylation (Lanes 9 and 10, respectively). A concentration of 100 μM 8-sulfophenyltheophylline caused a marked stimulation of 32P incorporation into the 31kD and 155kD proteins (data not shown). This stimulation was presumably the result of a theophylline-induced inhibition of a cardiac phosphodiesterase. Lanes 11 through 15 demonstrate that while PIA reduces the stimulatory effect of ISO (lane 14), when the more specific A1 antagonist DPCPX is present in conjunction with PIA and ISO (lane 13), the inhibitory effect of PIA is not observed. DPCPX in conjunction with ISO (Lane 12) produces a slight increase in phosphorylation when compared to ISO alone (Lane 11), however this tendency was not statistically significant.

[^32P]ATP SPECIFIC ACTIVITY, cAMP and PKA. The[^32P]ATP specific radioactivity, cAMP levels and PKA activity of the myocytes were examined (Table III) for
response to 0.1 μM ISO, in the absence or presence of 1 μM PIA or 10 μM CARB.

**TABLE III:** Substrate $\gamma^{32}$P]ATP specific radioactivity, cAMP content, and cAMP-dependent protein kinase activity of cardiac ventricular myocytes.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Spec. Act.</th>
<th>cAMP</th>
<th>PKA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.95 ± 0.24</td>
<td>4.3 ± 1.1</td>
<td>12.0 ± 0.6</td>
</tr>
<tr>
<td>ISO</td>
<td>4.15 ± 0.34</td>
<td>8.6 ± 1.0</td>
<td>32.3 ± 4.8</td>
</tr>
<tr>
<td>ISO/PIA</td>
<td>3.99 ± 0.10</td>
<td>8.3 ± 0.5</td>
<td>23.1 ± 1.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>ISO/CARB</td>
<td>4.07 ± 0.18</td>
<td>6.1 ± 0.4&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>22.0 ± 1.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
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Primary cultured myocytes were prepared and exposed to various agents as described in the Methods. $\gamma^{32}$P]ATP specific radioactivity (Spec. Act.) is expressed as cpm/pmol. cAMP levels are expressed as pmol/mg myocyte prot. Cyclic AMP-dependent protein kinase (PKA) activity is expressed as percent activation and reflects the activity ratios in the absence and presence of 2 μM cAMP x 100 as defined in Methods. ISO, PIA and CARB were used in 0.1 μM, 1 μM and 10 μM concentrations, respectively and administered as described in Figure 3. Results are the mean ± SE for 4-7 different experiments. The cAMP and PKA values were significantly different when compared to control. <sup>a</sup> indicates a significant difference when compared to ISO. <sup>b</sup> indicates a significant difference when compared to PIA with ISO.
To ensure that any variations seen in $^{32}$P incorporation were a result of the effect of a particular treatment rather than a change in substrate specific activity, the $[^{32}\text{P}]$ATP specific activity of myocytes treated with ISO (0.1 $\mu$M), or with a combination of PIA (1 $\mu$M) and ISO or CARB (10 $\mu$M) and ISO (inhibitor given 60 sec prior to ISO exposure). The specific activity remained constant with the various treatments. The cAMP level in the myocytes was also measured and found to be elevated 100% over control in the presence of ISO (0.1 $\mu$M, 30 sec). Although PIA had no apparent inhibitory effect on ISO-induced increases in cAMP formation, the stimulation of cAMP levels seen in the presence of ISO with CARB was significantly lower than that seen with ISO alone and represents a 58% inhibition of the ISO induced stimulation. The level of PKA activity was also measured and showed an increase in percent activation (as defined in Methods) of 170% over control values in the presence of ISO. In the PIA and CARB pretreated cells, the percent activation was reduced 46% and 51% respectively, relative to the PKA activity seen with ISO alone. Additionally, when PKA activity in the myocytes was measured in the presence of a specific cAMP-dependent protein kinase inhibitor (17) at a concentration of 25 $\mu$g/ml per assay tube, the activity induced by ISO was reduced to control levels (data not shown).
ISOPROTERENOL TIME COURSE SENSITIVITY TO PIA AND CARBA-CHOL. The effect of 1 μM PIA and 10 μM CARB on 0.1 μM ISO-induced $^{32}$P incorporation into 31kD and 155kD protein substrates varies according to the time of ISO exposure. The sensitivity of 0.1 μM ISO (15-120 sec ISO exposure) stimulated $^{32}$P incorporation to the effects of a 60 sec preexposure to either PIA (1 μM) or CARB (10 μM) is shown in Figures 10A and 10B. The inhibitory effects of CARB on the incorporation of $^{32}$P into the 31kD and 155kD proteins (an inhibition of 70% and 46%, respectively) appear earlier (15 sec, 10A and 10B) than do those of PIA. At the 30 sec time point, the inhibitory effects of PIA are seen, with inhibition of 37% and 25% seen for the 31kD and 155kD proteins, respectively. CARB produced inhibition of 47% and 35%, at this point. At the 60 sec time point, the ISO induced $^{32}$P incorporation into the 31 kD protein was inhibited 23% by PIA and 60% by CARB, with the $^{32}$P incorporation into the 155 kD protein being reduced 25% by PIA and 46% by CARB. By the 120 sec time point the most evident inhibition is that of a 33% reduction in ISO stimulated $^{32}$P incorporation of the 31kD protein in the presence of CARB. PIA, at the 120 sec time point, showed no effect on the ISO-induced increase in $^{32}$P incorporation into either protein substrate.
Figure 10. Isoproterenol time course sensitivity to inhibition by PIA or carbachol. The effect of a 60 sec pre-incubation with either PIA (1 μM) or carbachol (10 μM) on the 15-120 sec time course sensitivity of isoproterenol (0.1 μM) induced 32P incorporation into myocyte proteins. 155kD protein (6A), 31kD protein (6B). Results are expressed relative to the isoproterenol induced incorporation and represent the mean ± SE for 4-10 experiments. An asterisk denotes a significant difference compared to the ISO value alone. A dagger denotes a significant difference compared to the appropriate ISO with PIA value.
CONCENTRATION DEPENDENCE OF THE EFFECT OF PIA AND CARBA-
CHOL ON THE ISOPROTERENOL INDUCED $^{32}$P INCORPORATION INTO
PROTEIN SUBSTRATES. The concentration dependence of $^{32}$P incorporation was examined in myocytes preexposed (60 sec) to various concentrations of either PIA or CARB and then exposed to different concentrations of ISO for 30 sec. While CARB can be shown to always attenuate ISO-induced $^{32}$P incorporation, the inhibitory or stimulatory effect of PIA appears concentration dependent. The effect of various concentrations of PIA and CARB on the $^{32}$P incorporation induced by a 30 sec exposure to a range of ISO concentrations (1 nM to 10 μM) is shown in Figure 11. All samples treated with ISO alone were significantly different from untreated (control) cells at ISO concentrations above 10 nM. The effect of PIA on the ISO-induced increase in $^{32}$P incorporation in the 155kD and the 31kD proteins is shown in Figures 11A and 11B. At ISO concentrations greater than 10 nM (11A), PIA concentrations of 0.1 μM to 10 μM produced a significant reduction in the ISO-induced $^{32}$P incorporation into the 155kD protein. 100 μM PIA had no detectable effect on the ISO-induced $^{32}$P incorporation. In the 31kD protein (11B), at ISO concentrations greater than 10 nM, only PIA concentrations of 0.1 μM and 1.0 μM produced a significant reduction in $^{32}$P incorporation, with the exception of 1 μM PIA having no effect on 1 μM ISO. Although only 10 μM PIA reduced the effect of 10 μM ISO significantly, 100 μM PIA
produced a significant elevation in $^{32}$p incorporation at ISO concentrations of 0.1 and 1 µM.

Figure 11. Isoproterenol induced $^{32}$p incorporation dose response at selected concentrations of carbachol and PIA. The effect of PIA on the $^{32}$p incorporation into the 155kD and 31kD proteins, respectively (Panels A and B). The effect of carbachol (CARB) on the 155kD and 31kD proteins, respectively (Panels C and D). Results are expressed in relation to untreated (control) myocytes and represent the mean ± SE of 4-12 experiments. All myocyte preparations treated with ISO alone were significantly elevated above control myocytes by 149% to 822% for the 31kD protein and 146% to 238% for the 155kD protein at ISO concentrations above 0.01 µM. Significant differences are as indicated in the Results.
Figures 11C and 11D represent the reduction by CARB of ISO-induced $^{32}P$ incorporation into the protein substrates. In the 155kD protein (11C), at ISO concentrations above 10 nM, all concentrations of CARB significantly reduced $^{32}P$ incorporation, with the exception of 1 μM CARB which did not significantly decrease 10 μM ISO-induced $^{32}P$ incorporation. In the 31kD protein (11D), at ISO concentrations greater than 10 nM, 1 μM CARB significantly reduced $^{32}P$ incorporation at ISO concentrations of 10 nM and 0.1 μM, while CARB concentrations of 10 μM and 100 μM significantly reduced $^{32}P$ incorporation induced by ISO concentrations above 10 nM.

COMPARISON OF ISOPROTERENOL VERSUS FORSKOLIN INDUCED $^{32}P$ INCORPORATION. Forskolin was administered to the myocytes to examine the stimulation of protein phosphorylation through a non-β-adrenergic receptor mediated mechanism. While the $^{32}P$ incorporation can be in excess of 800% for both ISO and forskolin, it is evident (Figure 12) that on an equimolar basis, 10 μM ISO (30 sec) causes a more rapid increase in $^{32}P$ incorporation in the 31kD substrate than forskolin. The 31kD and the 155kD protein substrates show an increase in $^{32}P$ incorporation in response to both ISO (0.1 μM, 30 & 60 sec), and forskolin (10 μM, 30 & 60 sec; 1 μM, 300 sec) exposure. The increase seen in $^{32}P$ incorporation into both substrates produced by the higher concentration (10 μM) of forskolin is not inhibited by
either PIA or CARB, and may in fact be potentiated by these agents (2 of 5 experiments, data not shown).

![Bar chart showing percent 32P incorporation induced by isoproterenol and forskolin. Ventricular myocyte (32P labeled) protein response to agents (varied time course and concentration) known to produce inotropic changes in the heart. Results are expressed in relation to untreated (control) myocytes. All values are significantly different from the appropriate control values. An asterisk denotes a significant difference compared to the 30 s, 0.1 μM ISO value. Each treatment represents the mean ± SE of 4-10 experiments. ISO = isoproterenol, FSK = forskolin.]

Figure 12. Myocyte 32P incorporation induced by isoproterenol and forskolin. Ventricular myocyte (32P labeled) protein response to agents (varied time course and concentration) known to produce inotropic changes in the heart. Results are expressed in relation to untreated (control) myocytes. All values are significantly different from the appropriate control values. An asterisk denotes a significant difference compared to the 30 s, 0.1 μM ISO value. Each treatment represents the mean ± SE of 4-10 experiments. ISO = isoproterenol, FSK = forskolin.
However, the effect of 1 μM forskolin on the $^{32}$P incorporation into 31kD protein was attenuated 25% by PIA (1 μM) and 44% by CARB (10 μM), with the attenuating effect on the 155kD protein being 12% by PIA and 31% by CARB.

**SUBSTRATE IDENTIFICATION BY IMMUNOBLOTTING.** The 31kD protein substrate was found to specifically react with a monoclonal antibody to cardiac troponin I. The nitrocellulose transfer and autoradiograph of immunoblotting trials used to identify the 31kD protein are shown in Figure 13. The immunoblot in Figure 13a demonstrates a distinct protein band appearing at an approximate molecular weight of 31kD which corresponds to the single band at the same molecular weight in the autoradiograph shown in Figure 13B. The absence of any appreciable $^{32}$P incorporation in Lane a of the autoradiogram and the distinct $^{32}$P incorporation in lanes b and c indicates that the 31kD protein substrate is the cardiac regulatory protein, troponin I (TNI).
Figure 13. **Immunoblotting of the 31kD protein.** A nitrocellulose transfer blot (panel A), exposed to a monoclonal antibody (mouse) to skeletal muscle TNI, was developed using a secondary antibody (goat anti-mouse) and alkaline phosphatase system as detailed in Methods. The lowest arrow indicates a unique staining at a molecular weight of 31kD. The corresponding autoradiogram (panel B) of the nitrocellulose blot demonstrates a distinct band at 31 kD). Lane 1 represents control myocytes, while lanes 2 and 3 are myocytes exposed to 30 sec of 0.1 µM isoproterenol. In panel B, lanes a, b, and c correspond to lanes 1, 2, and 3 of panel A. The arrowheads indicate approximate molecular weights in ascending order of 31kD, 68kD, 155kD and 200kD. The typical ISO induced $^{32}P$ incorporation into the 155kD protein is seen in the upper region of panel B. The small points seen in the lower medial sections of both panels represent registration marks for alignment of the autoradiograph to the immunoblot.
DISCUSSION

Numerous studies in the perfused mammalian heart have demonstrated that the increase in the inotropic state produced by β-adrenergic agonists is clearly related to cAMP-dependent events resulting in the change of the phosphorylation state of several cellular proteins (33, 40, 109). It is also evident that the increases in inotropic state produced by β-adrenergic agonists are markedly attenuated by the presence of either adenosine or acetylcholine. In the perfused heart, both adenosine and acetylcholine reduce the catecholamine-stimulated increase in the $^{32}$P incorporation in several proteins in the myocardium (36, 87). The perfused heart unfortunately limits the resolution of biochemical events due to the presence of multiple cell types. While physiologically relevant, the antiadrenergic actions of adenosine and acetylcholine with regard to protein phosphorylation in the ventricular myocyte have not been extensively described.

This report presents, for the first time in the primary cultured rat ventricular myocyte, a comparison of the attenuating effects of adenosine and acetylcholine on the isoproterenol-induced increase in $^{32}$P incorporation into protein substrates, cAMP levels and PKA activity. Additionally, one of the protein substrates has been immunologically identified as the myofibrillar regulatory protein, troponin I.

With the improved techniques for obtaining viable
dispersed cell preparations from the myocardium, the study of the biochemical events occurring at the cellular level has been greatly facilitated (14,142). The ISO-induced increase in protein phosphorylation has been shown in the isolated ventricular myocyte in suspension (98,101) and in cultured cells (50).

ISO produces a time and concentration-dependent increase in protein phosphorylation, as evidenced by an increase in $^{32}$P incorporation into several protein substrates. As indicated in Methods, a change in $^{32}$P incorporation in a protein is routinely utilized as an index of a change in the phosphorylation state of a given substrate (25). Consequently, the term protein phosphorylation will be used interchangeably with $^{32}$P incorporation in discussion of these findings. While protein phosphorylation has been previously demonstrated in cellular suspensions (98,101), the method of using adherent myocytes presents additional evidence of physiological viability. Since it has been shown that non-adherent cells irreversibly degenerate in a few hours after isolation (64,121), concerns exist that observations made on myocytes in suspension may possibly be compromised by the metabolic decline of the cell. Furthermore, the concentrations and the time periods utilized herein were similar to those seen in earlier reports in the perfused heart (24,36,97), thereby permitting a comparison of a cellular event with a phenomenon observed in the perfused heart.
In the present investigation, no difference was seen in the inhibition of the ISO-induced $^{32}$P incorporation by PIA with adenosine deaminase present in the culture media. This is contrary to a previous report (58), and may imply that the myocytes used in this study produce little, if any, adenosine. As a result, endogenously produced adenosine by the myocytes does not appear to mask events examined in this investigation. Thus, the absence of endogenous adenosine also provides additional support for the isolated cardiomyocyte as a physiologically valuable model.

To better understand the significance of the protein substrates (31kD and 155kD) being affected differently by PIA and CARB, as well as the differing sensitivities to ISO, it is necessary to appreciate the role of these substrates in cardiac function. The 31kD protein was identified as TNI and it has been proposed that when phosphorylated this protein affects the sensitivity of TNC to calcium. Since this protein remains phosphorylated after contractility returns to normal, this explanation for the role of TNI has been questioned ((112). The 155kD substrate is believed to be C-protein (66) and thought to alter Mg-ATPase activity associated with the myofilaments. Like TNI phosphorylation, the changes in phosphorylation in C-protein accompanying β-adrenergic stimulation of the heart have not as yet been well correlated to changes in contractile function (46). It has even been suggested that
the degree of dephosphorylation of TNI and C-protein may be regulated by cholinergic stimulation in the heart (87).

The mechanism(s) underlying the greater relative potency of CARB as compared to PIA is as yet unclear. The reported three-fold greater density of the muscarinic versus the adenosinergic receptors in the rat ventricle (88) may be a component of the greater inhibitory effect seen with the higher concentrations of CARB. However, it is unlikely to be the sole explanation. The manner of coupling of the receptor to transmembranal elements, in addition to the type of cytoplasmic response elicited, may be different. While the antiadrenergic effects of both CARB and PIA are thought to be mediated through the GTP binding protein G_i, the proposed interaction of this protein with the catalytic subunit of adenylyl cyclase (9,55), and the mechanism(s) underlying the differences seen in response to CARB and PIA is unclear. Although, both agents reduce the ISO-induced increase in protein phosphorylation, only CARB is able to inhibit the increase in cAMP levels induced by catecholamine stimulation. It has been reported in the heart that muscarinic receptor stimulation produces an increase in IP_3, diacylglycerol (DAG) and cGMP levels (11,145). The specific roles these compounds may play in the antiadrenergic pathway is unknown. With the well described activation of calcium/phospholipid-dependent protein kinase C (PKC) by DAG, and the purported action of this enzyme on adenylyl
cyclase (5), modulation of this pathway by PKC should be considered. Additionally, the activation of a cGMP-regulated phosphatase by the increase in cGMP levels elicited by muscarinic stimulation could also have a role in the antiadrenergic function of carbachol (40).

The different roles of the A₁ and the A₂ receptors may also explain the concentration-dependent nature of the inhibition produced by PIA. With the A₁ receptor being inhibitory and of a higher sensitivity than the stimulatory, A₂ receptor, it is possible that the lower concentrations of PIA that produce the greatest inhibition of phosphorylation reflect primarily A₁ stimulation. The reduced inhibition produced by the higher concentrations of PIA could be the result of stimulation via the A₂ receptor in conjunction with inhibition through the A₁ receptor, with the result appearing simply as a reduction in inhibition at the higher concentrations. This is further supported by evidence that the higher doses of PIA stimulate phosphorylation in the myocyte.

It has been suggested (87) that a portion of the inhibitory effect of acetylcholine can not be explained by a reduction in cAMP levels but may be due to increased dephosphorylation of phospholamban. These proposed mechanisms of modulation between cAMP generation and PKA activation would provide pathways that may or may not involve G proteins. While it has been shown that the effects of both PIA and CARB are pertussis toxin sensitive (9,55),
the relationship between the adenosinergic and the muscarinic receptors and the interaction(s) of these receptors with $G_i$ is unclear. Whether the same molecule of $G_i$ is tied to both receptors types, or whether the two receptors interact with different isoforms or "pools" of $G_i$ remains to be determined and could impact upon the response of the myocyte when either or both of these agents are present.

An additional consideration resides in the concept of cellular compartmentalization or pooling of cAMP stores and enzymes in the myocyte. Intracellular compartmentalization of PKA has been suggested (14). Although PIA was not seen to produce an inhibition in the ISO-induced increase in cAMP, PIA was able to produce an attenuation in the level of PKA activity caused by isoproterenol, to a degree comparable to the inhibition produced by CARB. It is plausible that the overall level of cAMP remains relatively unchanged when the catecholamine-challenged heart is treated with PIA, yet a particular compartment of cAMP, with direct access to a specific component of a sub-cellular pool of PKA, could be preferentially reduced by the action of PIA. This process could serve as a conveyance of the inhibitory function without effecting global levels of cAMP in the cell. The concept of sub-cellular compartmentalization is further supported by the observation that cAMP levels produced by forskolin are markedly increased over those seen with isoproterenol (33). However, in such experiments the activation of PKA and protein phosphoryla-
tions elicited by either of these agents were similar, despite the significantly smaller change in cAMP caused by ISO.

In addition to the ability of CARB to produce a greater inhibition of ISO-stimulated protein phosphorylation than PIA, it was noted that inhibitory effects of CARB were seen at an earlier time point of ISO exposure and that this inhibition was seen over a longer period of ISO exposure than the inhibition produced by PIA. This rapidity of action by CARB (relative to the time course of PIA) could be due to a different sensitivity to CARB versus PIA. However, this response could also suggest different interactions between the transmembranal components of the β-adrenergic system and the muscarinic system versus the adenosinergic system.

The increase in protein phosphorylation induced by forskolin is of interest for several reasons. With an established effect in the perfused heart (33), the use of forskolin was initially intended to demonstrate a mechanism, other than β-adrenergic receptor mediated, by which protein phosphorylation could be stimulated in the myocyte. Since the site of action of forskolin in the myocyte was believed to be directly upon the catalytic subunit of the adenylyl cyclase complex (124), it was thought that forskolin stimulation would not be inhibited by either PIA or CARB. While at higher concentrations of forskolin no inhibition by PIA or CARB was noted, the
stimulation of protein phosphorylation seen with lower concentrations of forskolin was inhibited by PIA and carbachol. This observation supports recent findings in primary cultured myocyte membrane preparations (116) that PIA inhibition of forskolin-stimulated adenylyl cyclase activity occurs, over a narrower range of forskolin concentration, and to a lesser degree than did the inhibition by PIA of ISO-stimulated adenylyl cyclase activity. This raises a question concerning the mechanism(s) of action of forskolin. While little is understood about the action(s) of forskolin, it has been suggested that the myocyte may possess a forskolin receptor (119), a factor which may serve as a component of the concentration-dependent sensitivity of forskolin to PIA and CARB by providing a site in addition to the purported direct site of action of forskolin on the catalytic subunit of adenylyl cyclase (140). It has also been suggested that forskolin may interact directly with the G protein (146). Should forskolin present multiple sites of action or multiple affinities for the same site, this inotropic agent may be included in the list of agonists susceptible to the effects of certain antiadrenergic agents.

The identification by immunoblotting of the protein phosphorylated by ISO provides strong evidence that this protein is a component of the myofilament system of the myocyte. Both TNI and C-protein have been shown to be substrates of PKA, as well as other kinases associated
with the myocardium (5,33,40). Although the function of these proteins in the myocardium is not well understood, their association with the contractile apparatus and their covalent modification by catecholamine induced phosphorylation may imply a physiological role (25,36,46). Additionally, when the long term period of dephosphorylation of these proteins is contrasted to the more rapid dephosphorylation reported for phospholamban (40), a role in the contractile process may be envisioned. In that TNI and C-protein are known to be phosphorylated in the catecholamine treated heart, and the period of increased phosphorylation lasts well after the removal of the catecholamine (40), these proteins may have a role in developing a "preferential or permissive state" for a rapid change in inotropy. In this preferential state, the more rapid adjustments in inotropic state would be the result of proteins such as phospholamban, controlling real-time calcium levels and thereby adjusting "beat to beat" inotropic state. This would be consistent with the observation that both TNI and C-protein remain phosphorylated long after the increase in inotropic state produced by catecholamines is diminished by the removal of the catecholamine (123).

In summary, this investigation has demonstrated that troponin I and a 155kD protein, possibly C-protein, are phosphorylated in the presence of ISO in the myocyte and that these phosphorylations are attenuated by the
adenosinergic agonist, PIA, as well as the muscarinic agonist, carbachol. Additionally, it was shown that CARB produces a greater inhibitory effect on the ISO induced phosphorylation of these proteins than PIA. Forskolin has also been shown to produce similar increases in phosphorylation, with PIA and carbachol able to reduce these increases, although to a lesser degree than the reduction of ISO-induced increases in phosphorylation. While a major component of the antiadrenergic properties of these agents is felt to be mediated through the transmembrane protein, Gᵢ, the degree to which carbachol more effectively inhibits ISO-induced protein phosphorylation suggests a more complex mechanism of inhibition to perhaps include multiple sites of action.
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CHAPTER V

CONCLUSION

The regulation of myocardial contractility and metabolism involves mechanisms unique to cardiac muscle as well as those common to all muscle types. A primary mechanism common to all types of muscle involves intracellular transduction and amplification of signals by reversible covalent modification of proteins by phosphorylation. This mechanism of modification, resulting from a conformational change in protein structure brought about by phosphorylation, can produce either an activation or an inhibition of selected protein function.

Studies of cyclic oscillations of cAMP-related events during the contraction cycle. Since the basic mechanism of muscle contraction is known to involve changes in the phosphorylation state of cellular proteins (141), the nature of these covalent modifications and the manner by which these changes are brought about appear to be an integral part of the regulation of myocardial metabolism and contractility. To better understand the role of protein phosphorylation in the regulation of cardiac contractility and metabolism, the myocardium was initially examined in view of the uniquely cyclic property of contraction in the heart. A device capable of arresting the perfused rat heart in less than 50 milliseconds (134) was
utilized to investigate changes in cAMP and in the activity of 2 cAMP-dependent enzymes during the cardiac contraction cycle. By measuring these parameters at different points (within the cycle) we could determine whether or not cAMP-dependent changes underlie metabolic events that occur during contraction.

The results from these studies revealed no changes in cAMP levels, or in the activities of cAMP-dependent protein kinase or glycogen phosphorylase during a single contraction cycle. This finding conflicts with earlier reports suggesting that cyclic AMP levels and glycogen phosphorylase activity change as a function of the contraction cycle (134,144). However, the nature and degree of the changes reported, in addition to the experimental designs utilized, present certain concerns as to the physiological significance of such findings. For example in one of these studies employing perfused rat heart, myocardial tissue was subjected to field stimulation and such conditions may have evoked release of endogenous catecholamine (10). In another investigation in the rat (134) the variation in glycogen phosphorylase activity during the contraction cycle was examined at lower than physiological temperatures and in the presence of mechanical and pharmacological interventions to elongate the single contraction cycle. Other studies that demonstrated cyclical changes in cAMP, ATP, ADP, glucose-6-phosphate, pyruvate, phosphocreatine, and creatine employed nanomolar
levels of isoproterenol, high calcium and glucose in the perfusate, coupled with increased afterload pressure. These changes were not observed in pyruvate perfused rat hearts (141). In another study on metabolic change during the cardiac cycle in the dog heart transient changes in cAMP levels and in the activities of cAMP-dependent protein kinase and glycogen phosphorylase were noted during the contraction cycle. However, propanolol treatment abolished the fluctuations in cAMP and cAMP-dependent protein kinase, while having no effect on the fluctuations demonstrated by glycogen phosphorylase (78). Thus it seems likely that the variation in cAMP and cAMP-dependent protein kinase results from a beat to beat fluctuation in endogenous catecholamine release.

These technical concerns force us to conclude that more rigorous tests must be employed to demonstrate that oscillatory changes in energy-related events occur in the functioning myocardium. While certain events such as calcium flux (83) and conformational changes in the contractile proteins (70) are known to occur in a cyclic manner during the contraction cycle, the oscillatory nature of other events, such as cAMP, ATP, and ADP levels and additional elements associated with myocardial metabolism are not as clearly understood. The cyclic manner of myocardial contraction suggests that certain biochemical events must oscillate during a single heart beat. However, the extremely short time course of contraction in the
mammalian heart, and the fact that a subtle biochemical change might evoke a marked physiological effect in myocardial function, make this technically difficult. Additionally, it is possible that cyclic changes occur only in specific sub-cellular compartments such that these changes are obscured when analyses measure the levels of metabolites in whole cells or organs.

The existence of compartments, while difficult to demonstrate, is readily inferred. Although forskolin will increase myocardial cAMP levels to much higher concentrations than those evoked by isoproterenol, the activation of cAMP-dependent protein kinase by isoproterenol occurs more rapidly and to a greater extent than the activation by forskolin (33). Prostaglandin E₁ produces an elevation in cAMP levels and activates cAMP-dependent protein kinase in the heart. However, these increases do not appear to effect the contractile state of the heart (51). To explain the differences in response to these agents (isoproterenol, forskolin, prostaglandin), it has been suggested that ISO but not forskolin or PGE elicits an increase in cAMP in a sub-cellular compartment(s) of cAMP that has preferential access to cAMP-dependent protein kinase. Since a change in contractile state is only observed with isoproterenol and forskolin, it is evident that protein phosphorylation is integral in the mechanism.

Cyclic AMP-dependent and calcium/phospholipid-dependent protein kinases translocate from particulate to
soluble and from soluble to particulate fractions of a cell, respectively, during catecholamine stimulated activation (53). This suggests that elements of a cell may occupy a certain compartment or domain within the cell and, upon activation or stimulation, may relocate to a different compartment to perform a given role.

Chapter Selected Protein Kinases and Substrates in the Myocardium. Protein kinases as cellular effectors in the myocardium provide a mechanism of translation of extracellular and intracellular signals into intracellular responses. In addition to the role as intracellular mediators, protein kinases also provide potential sites of regulation. The activities of cAMP-dependent, calcium/calmodulin-dependent, calcium/phospholipid-dependent and cGMP-dependent protein kinases determined in a canine cardiac sarcolemmal preparation demonstrate the relative influence of these enzymes, with cAMP-dependent protein kinase being the most active. Additionally, these kinases were found to phosphorylate endogenous protein substrates in this preparation with particular substrates phosphorylated by 2 or more kinases. Since cAMP-dependent protein kinase had the greatest activity and since it produced the greatest degree of endogenous protein phosphorylation, it seems likely that cAMP-dependent protein kinase exerts the most influence with respect to myocardial metabolism.
Since the kinases examined appear to act on the same substrates, it would be of interest to determine whether these enzymes act in a complimentary or related manner. Cyclic AMP-dependent protein kinase may provide the principle or initiating effect by phosphorylation of a substrate. Another kinase, such as calcium/calmodulin-dependent protein kinase may then serve to intensify or prolong the effect produced by covalent modification of a given substrate. This interdependence could also provide a means for different second messengers, such as cAMP and calcium, to produce similar or complimentary effects through different cellular pathways. With the change in cAMP-dependent protein kinase isoforms that occur in the diseased myocardium (105), it is conceivable that a second kinase, such as calcium/calmodulin-dependent kinase, may increase in activity to counter a change in cAMP-dependent protein kinase activity. This overlap in enzyme function could provide a reserve of kinase activity. This reserve could also serve to limit the effect of the saturation of a given kinase and increase the rate of response of a particular system by (simultaneous) activation of multiple kinases.

Since cAMP-dependent protein kinase, calcium/phospholipid-dependent protein kinase and calcium/calmodulin-dependent protein kinase have been shown to have specific actions at the level of the sarcolemma (63,106,138), the probability of these kinases being present as a result of
a preparational artifact appears minimal. Cyclic AMP-dependent protein kinase, probably the most closely examined in the myocardium has been implicated in the shown phosphorylation of a protein substrate that is closely associated with the sarcolemmal calcium channel (125). Additionally, electrophysiological investigation has demonstrated a direct correlation between the presence of the catalytic subunit of cAMP-dependent protein kinase and an increase in calcium flux through the voltage sensitive calcium channel (69, 147). Calcium/phospholipid-dependent protein kinase and calcium/calmodulin-dependent protein kinase phosphorylate a cytoplasmic face sarcolemmal protein (63).

Since changes in phosphorylation states of protein substrates alter myocardial metabolism and contractility, understanding the particular proteins that are phosphorylated and the manner in which these phosphorylations are regulated could provide a more direct appreciation of kinase-related cellular events in the myocardium.

Protein Phosphorylation in the Isolated Myocyte. While the perfused heart provides an excellent means for comparing of functional parameters of the working heart with biochemical changes in the organ, the multiple cell types present in the organ eliminate the possibility of ascribing a particular change to a specific cell type. Viable dispersed ventricular myocytes offer the opportunity to discern the specific metabolic events that occur in cardi-
ac cells under various physiological conditions. These conditions led us to reexamine the effects of catecholamine stimulation on protein kinase activity and protein phosphorylation in ventricular myocytes and to assess the effects of acetylcholine and adenosine on β-adrenergic response.

These studies demonstrated that β-adrenergic stimulation of ventricular myocytes elicits an increase in cAMP levels and in cAMP-dependent protein kinase activity that results in phosphorylation of phospholamban, troponin I and a protein felt to be C-protein in the isolated ventricular myocyte, changes similar to those described previously in the perfused heart (40). When myocytes are pretreated to the antiadrenergic agents carbachol and phenylisopropyl adenosine, prior to the introduction of catecholamine, the effects of the catecholamine are in general attenuated. Exposure of the myocytes to the diterpene, forskolin, produces somewhat larger increases in cAMP but comparable changes in contractility as compared to the case with catecholamines, a finding similar to that seen in the perfused heart (33).

There is an interesting exception to the antiadrenergic role of carbachol and phenylisopropyl adenosine in the myocyte when compared to that seen in the perfused heart. While carbachol and phenylisopropyl adenosine attenuate catecholamine-induced effects in the perfused heart, the nature and scope of the effect of phenylisoprop-
pyl adenosine in the isolated myocyte vary in relation to concentration. At lower concentrations of adenosine, the effect of catecholamines on cAMP-dependent protein kinase and protein phosphorylation in the heart are reduced, while cAMP levels are unchanged from those levels seen with catecholamine stimulation. At elevated levels of phenylisopropyl adenosine, catecholamine-induced increases in protein phosphorylation appear to be potentiated.

The opposite effects of phenylisopropyl adenosine on catecholamine-induced events in the myocyte may be explained by the differences in the two different adenosine-ergic receptor subtypes present in the myocardium. The A<sub>1</sub> subtype, presumably coupled to adenylyl cyclase via the nucleotide binding protein G<sub>i</sub>, inhibits adenylyl cyclase activity and this inhibitory effect is seen at relatively low concentrations of adenosine (K<sub>d</sub> 1.2 nmol for PIA). The A<sub>2</sub> subtype, presumably coupled to adenylyl cyclase via G<sub>s</sub> stimulates adenylyl cyclase, but relatively high concentrations of adenosine (K<sub>d</sub> 120 nmol for PIA) are required to elicit this response (116). Consequently, lower doses of phenylisopropyl adenosine are inhibitory with regard to catecholamine-induced effects in the myocyte, while higher phenylisopropyl adenosine concentrations produce stimulatory effects with regard to adenylyl cyclase that may be complimentary to those produced by the catecholamine.
To understand why a single agonist interacts with two different receptor subtypes to elicit opposite effects in the same cell type, one needs to appreciate the dynamics of adenosine metabolism in the myocardium. With catecholamine stimulation, the level of adenosine increases due to the increased metabolic activity. This rise in adenosine acting via A1 receptors can act as a feedback inhibitor, limiting the catecholamine-induced increases in metabolism (22,23). This is suggested by the increase in catecholamine stimulation of the oxygenated heart increasing adenosine levels and possibly preventing over stimulation of the heart (28). If catecholamine stimulation becomes excessive as may occur in the failing heart (105), the correspondingly higher adenosine levels (26) may activate A2 receptors, potentiating catecholamine action. This mechanism could be envisioned as a final effort to maintain a minimal level of mechanical activity by a diseased organ.

A second difference regarding the antiadrenergic actions of carbachol and phenylisopropyl adenosine involves the influence of these agents on catecholamine-induced increases in cAMP levels. Although carbachol and phenylisopropyl adenosine both reduce PKA and PKA-dependent protein phosphorylation, only carbachol reduces the increase in cAMP levels evoked by catecholamines in the myocyte. This difference may be attributable to the additional effects of muscarinic agents to increase dia-
cylglycerol and IP₃ release, and stimulate phosphodiesterase activity (95,145). PIA, on the other hand has no known effect on PI turnover although it inhibits adenylyl cyclase (84). The finding that PIA failed to attenuate catecholamine-stimulated cAMP levels in the myocyte differs from the findings in perfused heart. The explanation for this discrepancy is unclear and may reflect preparational differences or an effect on phosphodiesterases in the myocardium since high levels of adenosine analogues inhibit phosphodiesterase (95). Such an inhibition could mask a localized change in cAMP levels evoked by phenylisopropyl adenosine. Thus, the contrasting effects on phosphodiesterase activity of these antiadrenergic agents may provide a basis for the differing effects regarding isoproterenol-induced cAMP levels.

IV. Continued Studies. Since carbachol and adenosine have different effects on phosphoinositol turnover, it is possible that phosphoinositol metabolism represents another inhibitory pathway that leads to the reduction in cAMP seen in the presence of carbachol. Since diacylglycerol activates protein kinase C (131), the antiadrenergic effects of carbachol could be in part mediated by this protein kinase. Additionally, subcellular compartmentalization may underlie the different actions of these two agonists. Phenylisopropyl adenosine may act to inhibit a small pool of adenylyl cyclase in a compartment with preferential access to cAMP-dependent protein kinase,
while carbachol may interact with a larger pool of cyclase such that it produces more marked changes in total cellular cAMP, only a small fraction of which may be involved in regulating cAMP-dependent protein kinase. Also, the ability of carbachol to stimulate phosphodiesterase activity (8) may contribute to the reduction in cAMP levels. This effect of carbachol on phosphodiesterase activity is in contrast to the effects of high concentrations of phenylisopropyl adenosine which inhibit phosphodiesterase activity in the perfused heart (95).

The speculation concerning the differential effects regarding cAMP levels of these two antiadrenergic agonists may extended to be consider the manner in which the muscarinic and the adenosinergic receptors are coupled to the membrane bound adenylyl cyclase (116,145). Whether there is a preferential coupling of the muscarinic receptor or a greater number of muscarinic receptors linked to the adenylyl cyclase is unclear. Since the manner of interaction within the receptor, G protein, adenylyl cyclase complex is not well understood for either the muscarinic or adenosinergic receptors, the relative differences in their inhibition of adenylyl cyclase is as yet unexplained. Further examination (88) to correlate receptor densities (A₁ vs M₂), with the relative effects of equimolar amounts of phenylisopropyl adenosine and carbachol would provide insight regarding the relative efficacies of these antiadrenergic agents.
Significant attention has been directed toward transmembranal events associated with adrenergic and antiadrenergic activity (123). A complimentary approach to understanding antiadrenergic mechanisms may lie in examining intracellular responses to antiadrenergic agents. In this manner a change in a cellular component, such as a kinase or a phosphoprotein could be associated with a particular agonist.

Phenylisopropyl adenosine and carbachol inhibit the isoproterenol-induced increase in the phosphorylation of certain protein substrates in the heart, presumably by reducing cAMP-dependent protein kinase activity. Carbachol also produces an elevation of diacylglycerol and cGMP levels in the heart (145), known activators of calcium/phospholipid-dependent protein kinase and cGMP-dependent protein kinase, respectively (43,131). These additional effects of muscarinic agonists (when compared to the effect of phenylisopropyl adenosine) may be a basis for the different degree and manner of antiadrenergic response produced by these agonists. Investigation of the action of these kinases in the presence of a selective inhibitor to cAMP-dependent protein kinase to identify cellular substrates and intracellular domains could provide information to differentiate between the actions of phenylisopropyl adenosine and carbachol. In this manner it may be possible to identify the nature and extent of modulation of various cellular elements by these agents.
Since catecholaamine stimulation leads to a translocation of cAMP-dependent protein kinase from the particulate or membrane associated sub-cellular fraction to the cytoplasmic or soluble fraction (53) it seems likely that cellular compartmentalization or regionalization may exist in the myocyte (14). This partitioning may also be evident regarding the distribution of certain protein kinases and intracellular messengers in the myocyte, and it is possible that further examination could reveal differential effects of agonists based on access to various compartments.

A preliminary test of compartmentalization in the myocyte could be performed using existing techniques. Differential centrifugation of $^{32}$P labelled myocytes treated with catecholamines in the presence and absence of carbachol or phenylisopropyl adenosine should provide strong indication of any regionalization of agonist effect. Autoradiographic analysis of cellular proteins, determination of protein kinase activities and the levels of intracellular messengers would permit the construction of a cell map. In this manner variations in quantities, locations of cellular effectors and the correlation of any changes to structures within the myocyte could be expressed as a function of sub-cellular locale.

The multiple effects of the cardiac muscarinic receptor (145) could be examined in view of the activation of diacylglycerol release, the reduction in
catecholamine-stimulated cAMP levels and the stimulation of cGMP in the myocyte. Since DAG and cGMP are known to activate PKC and cGMP-dependent protein kinase, changes in the activity of these enzymes under the conditions mentioned earlier could provide an insight to the role of non-cAMP-dependent pathways in the antiadrenergic role of carbachol.

The different effects of the adenosinergic receptors could also be examined in a similar framework. Currently available A₁ and A₂ agonists and antagonists could be used to selectively observe the response to each adenosinergic receptor subtype. In view of the stimulatory nature of the A₂ with respect to adenylyl cyclase, it is possible that the antiadrenergic effects of phenylisopropyl adenosine may not be completely understood since the A₁ response may be damped (dampened) by an A₂ mediated component. While these two opposing systems may serve synergistically in vivo, a better understanding of underlying mechanisms could result from the ability to examine each subtype in the absence of the other. Using an A₂ specific antagonist, it would be possible to strictly examine the A₁ elicited effects of phenylisopropyl adenosine. Similarly, the use of an A₁ antagonist would allow examination of the stimulatory effects of adenosine. Combining the A₁ antagonist with an inhibitor of protein kinase A could demonstrate the degree of similarity between A₂ and β₁ mediated stimulation of adenylyl cyclase.
With a more complete appreciation of the individual functions of these antiadrenergic activities in the myocyte, it would be possible to consider the manner in which these elements may interact to modulate myocardial metabolism and contractility.
APPENDIX

MYOCYTE ISOLATION: Isolated, calcium tolerant ventricular myocytes were prepared as previously described (Romano, 89, AJP). In brief, hearts were excised from decapitated adult male rats (250-350g) and immediately perfused at 37°C via an aortic cannula at constant pressure (70 mm H₂O) using physiological saline (PSS) containing in millimolar amounts; 119.4 NaCl, 4.7 KCl, 25 CaCl₂, 10 glucose, 25 NaHCO₃, 1.2 MgSO₄ and 1.2 KH₂PO₄. The pH was maintained at 7.4 by constant gassing with 95% O₂-5% CO₂. After an equilibration period of approximately 12 minutes, the hearts were stopped by means of a 60 second period of perfusion with calcium free PSS, whereupon a solution containing 0.80% collagenase, 0.30% hyaluronidase, 0.10% bovine serum albumin (BSA) and 50 μM CaCl₂ was perfused at a constant flow rate of 3.5 ml per minute per heart. At the first sign of surface striations (usually within 7-10 minutes), the ventricles were removed from the cannula, longitudinally quartered and placed in a flask containing 0.80% collagenase, 0.30% hyaluronidase, 0.25% BSA and 50 μM CaCl₂ for gentle agitation at 37°C (40 cycles/min) while gassed with 95% O₂-5%CO₂. The media was gently removed by aspiration every 7 min and replaced with fresh media for a total of 3-5 changes per preparation. When the sections began to appear soft and stringy and tore
easily when held by forceps, the media was again changed and the flask was rapidly agitated, under the same conditions as noted before, for 10 min. The contents of the flask was then strained through a nylon mesh (pore size 250 micron), washed with a solution of 0.60% BSA and 100 μM CaCl₂ (WASH) and allowed to settle at room temperature for 15 min. The upper two-thirds of the WASH was gently removed by aspiration and replaced with 40 ml of fresh WASH for a second 15 min settling. The WASH solution was removed as close to the pellet as practicable and replaced with 40 ml of media M199 (Gibco: 25 mM NaHCO₃, 25 mM HEPES, EARLE'S salts) for an additional settling of 10 min. The supernatant was removed and replaced with fresh M199 of a volume to permit plating on polystyrene culture dishes at an approximate final density cell density of 20-30 cells per high power (300X) microscope field.

Culture dishes were preincubated for 4 hours with M199 supplemented with heat inactivated fetal calf serum (FCS, Gibco) to provide a final concentration of approximately 4% FCS. Dishes were maintained from the pre-incubation phase through completion of the experiment in a humidified, quick recovery incubator (37°C) under constant gassing with a 5% CO₂-room air mixture. Preparations that did not yield a minimum of 75% rod shaped cells as determined by microscopic inspection at final settling were discarded.
References


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