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Anti-fibrotic effects of adenylyl cyclase in the heart: implications for connective tissue remodeling by cardiac fibroblasts

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy

in

Molecular Pathology

by

James S. Swaney

Committee in charge:

Professor Paul A. Insel, chair
Professor Sylvia M. Evans
Professor James R. Feramisco
Professor David M. Roth
Professor Francisco J. Villarreal

2005

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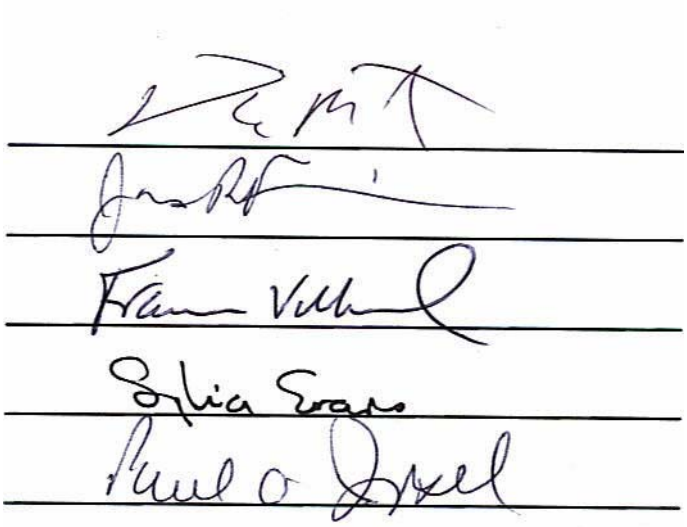

Chair

University of California, San Diego

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LIST OF ABBREVIATIONS

CHF	congestive heart failure
EDD	end-diastolic dimension
CF	Cardiac fibroblast
AC5	adenylyl cyclase type 5
AC6	adenylyl cyclase type 6
SH2	src homology 2
FAK	focal adhesion kinase
GPCR	G protein-coupled receptor
ECM	extracellular matrix
MI	myocardial infarction
TGFβ	transforming growth factor-β
IL1-β	interleukin 1β
IL-6	interleukin 6
TNFα	tumor necrosis factor-α
Ang II	angiotensin II
LV	left ventricular
MMP	matrix metalloproteinase
TIMP	tissue inhibitor of matrix metalloproteinase
CTGF	connective tissue growth factor
PAI-1	plasminogen activator inhibitor 1
α-SMA	α-smooth muscle actin
FA	focal adhesion
cAMP	cyclic adenosine monophosphate
Fsk	forskolin
AM	adrenomedullin
CaM	calcium/calmodulin
PKA	protein kinase A
PKC	protein kinase c
NO	nitric oxide
PDE	phosphodiesterase
PGE_2	prostaglandin E2
% FS	% fractional shortening
IP	intraperitoneal
DMEM	Dulbecco's modification of eagle's media
FBS	fetal bovine serum
TCA	trichloroacetic acid
IBMX	isobutylmethylxanthine
PTP	protein tyrosine phophatase
MES	2-(4-morpholino)-Ethane Sulfonic acid
BSA	bovine serum albumin

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VITA

1995 B.A., University of California, Santa Barbara

2002 M.S., University of San Diego

2005 Ph.D., Universtiy of California, San Diego

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ABSTRACT OF THE DISSERTATION

Anti-fibrotic effects of adenylyl cyclase in the heart: implications for connective tissue remodeling by cardiac fibroblasts

by

James S. Swaney

Doctor of Philosophy in Molecular Pathology

University of California, San Diego, 2005

Professor Paul A. Insel, Chair

Cardiac fibroblasts (CF) are the most abundant cell type in the heart and are responsible for extracellular matrix (ECM) homeostasis and repair in response to cardiac injury. Following myocardial insult, exaggerated or maladaptive production of ECM material results in myocardial stiffening and decreased cardiac relaxation, ultimately leading to diastolic heart failure. A key component of increased collagen production by CF is their transformation from quiescent fibroblasts, into activated myofibroblasts, which are characterized by expression of α-smooth muscle actin (α-SMA) and an increased collagen production capacity. Adenylyl cyclases (AC) are membrane-bound proteins that catalyze conversion of ATP to cyclic AMP (cAMP), a ubiquitous second messenger that influences growth, death, and differentiated cellular functions. Previous reports indicate that AC activation, or increases in cAMP, inhibit collagen production by CF, however no studies have examined the role of AC in CF function after myocardial infarction (MI) or examined the effects of AC activation on

cardiac myofibroblast formation. In this thesis, I investigated AC function by CF isolated from adult rats after MI and conducted subsequent studies to determine the effects of AC activation and overexpression on myofibroblast transformation and collagen production. Furthermore, I examined the cellular compartmentation of AC with other catalytic and structural molecules in CF in order to elucidate the cellular mechanisms by which cAMP regulates fibroblast-myofibroblast transformation and collagen production.

Chapter 1

Introduction

Congestive heart failure (CHF) is a leading cause of morbidity and mortality in the US and currently affects nearly 5 million people (see http://americanheart.org). CHF is functionally defined as a condition in which the heart is unable to pump enough blood to meet the metabolic demands of the body [1]. Initially, CHF was believed to result almost exclusively from systolic dysfunction, or more specifically, an inability of the heart to pump blood. More recently, it has been recognized that diastolic dysfunction, or the inability of the heart to fill with blood during diastole, is the underlying mechanism in a large proportion of heart failure cases [2]. Diastolic heart failure accounts for 30% to 50% of heart failure in the clinical practice. Diastolic dysfunction is caused by several factors that impede the active or passive phases of diastole, including ventricular hypertrophy, myocyte impairment, and increased interstitial fibrosis [2].

Myocardial fibrosis is one of the key pathological features of myocardial remodeling in CHF. Cardiac fibrosis reflects hyperplasia and altered production of connective tissue proteins and is characterized by an exaggerated deposition of extracellular matrix (ECM) material, predominantly collagen types I and III, into the interstitial and perivascular space [3-5]. Increased collagen deposition results in myocardial stiffening and decreased relaxation of the heart, ultimately leading to diastolic dysfunction and heart failure [3, 6]. In addition to deleterious effects on cardiac function, cardiac fibrosis also leads to abnormalities of coronary reserve and electrical activity, dramatically impacting the clinical outcome of heart failure patients [1, 4]. Regardless of the underlying etiology, the factors leading to heart failure

ultimately result in diminished contractile performance and pathophysiological remodeling [3].

The normal myocardium is composed of cardiac myocytes and non-myocytes, which include endothelial cells, vascular smooth muscle cells and fibroblasts [7]. Cardiac fibroblasts (CF) are the most abundant cell type in the heart, constituting two-thirds of the total cell population [3, 8]. In the normal heart, CF are responsible for extracellular matrix (ECM) homeostasis, and create the scaffold for cardiac myocytes and other cell types. In response to myocardial injury, CF also play a critical role in repair of the injured myocardium [3]. Following myocardial infarction (MI) or other cardiac insult, fibroblasts migrate into the region of injury where they proliferate and replace necrotic myocytes [1]. Fibroblasts within the wound area then become activated in response to increased mechanical tension, as well as various autocrine/paracrine stimuli, and begin to synthesize ECM material to produce a mature scar [9]. In addition to scar formation at the site of injury, fibroblasts remote from the infarct zone can also become activated thereby producing exaggerated amounts of ECM in the remaining, functional regions of the myocardium. This “remote fibrosis” reduces contractile performance resulting in subsequent diastolic dysfunction and heart failure [10-12].

Various fibrogenic growth factors such as angiotensin II (ang II) and transforming growth factor β (TGFβ) and inflammatory cytokines, including tumor necrosis factor α (TNFα) and interleukins 1β (IL-1β) and 6 (IL-6) have been identified as important mediators of cardiac collagen production and degradation. Ang

II is a critical mediator of cellular changes associated with left ventricular hypertrophy, remodeling post-myocardial infarction (MI) and heart failure. Ang II generation [13, 14] and ang II receptors are increased in the rat heart following myocardial infarction. Ang II also exerts profibrotic effects on fibroblasts isolated from explanted human hearts [15]. Studies of both neonatal and adult rat cardiac fibroblasts have shown that Ang II stimulates collagen synthesis [16-18]. These profibrotic effects of Ang II are mediated, in large part, via TGFβ [19].

Transforming growth factor β (TGFβ) is a highly potent pro-fibrotic factor in the heart, as well as other tissues, and contributes not only to infarct scar formation but also to interstitial fibrosis that appears remote from the infarct site [20-22]. In the adult rat, TGFβ mRNA is markedly increased following MI [11, 21] and TGFβ administration causes a dose-dependent increase in collagen production by CF [20]. TGFβ suppresses overall proteolytic degradation of ECM through regulation of matrix metalloproteinases (MMP's) and tissue inhibitors of metalloproteinases (TIMP's) [23] and stimulates the expression of proteins such as connective tissue growth factor (CTGF), plasminogen activator inhibitor (PAI-1) and IL-6; factors that are also linked to enhanced ECM production [24] [25]. Upon binding of TGFβ to receptors on the cell membrane, TGFβ-signaling is mediated by effector Smad proteins [26] that translocate to the nucleus and activate expression of target genes [27] involved in fibroblast proliferation, differentiation and ECM synthesis.

Inflammatory cytokines are also implicated in ECM remodeling in the heart though both positive and negative regulatory mechanisms. TNFα contributes to early

cardiac remodeling through stimulation of MMP activity leading to increased ventricular dilatation and pump dysfunction [28, 29]. Similarly, IL-1β increases total MMP activity in neonatal and adult rat fibroblasts, concomitant with decreased collagen synthesis [30]. Although expression of IL-6 is stimulated by TGFβ [25], suggestive of a profibrotic role for IL-6 in the heart, the IL-6 family member, leukemia inhibitory factor (LIF), [31] appears to exert antifibrotic effects on cardiac fibroblasts. Overall, these data suggest highly variable roles for inflammatory cytokines in cardiac remodeling and ECM regulation.

A key component of increased ECM deposition by CF is their transformation from a quiescent cell, the fibroblast, responsible mainly for ECM homeostasis, to an activated cell type, the myofibroblast, which plays a dynamic and central role in healing of the injured myocardium [3]. Myofibroblasts are a unique group of smooth-muscle-like fibroblasts that have a similar appearance and function regardless of their tissue of residence [32], and are characterized by the presence of α-smooth muscle (SM) actin, the most reliable marker of myofibroblastic cells [33]. In addition to their normal role in tissue homeostasis and repair, altered number and function of myofibroblasts are implicated in diseases with increased extracellular matrix (ECM) deposition and resultant fibrosis, such as those involving liver, skin, lung, and kidney [34-36]. In fact, modulation of the fibroblastic cell toward the myofibroblast phenotype is believed to be essential for connective-tissue remodeling during normal and pathological wound healing [9].

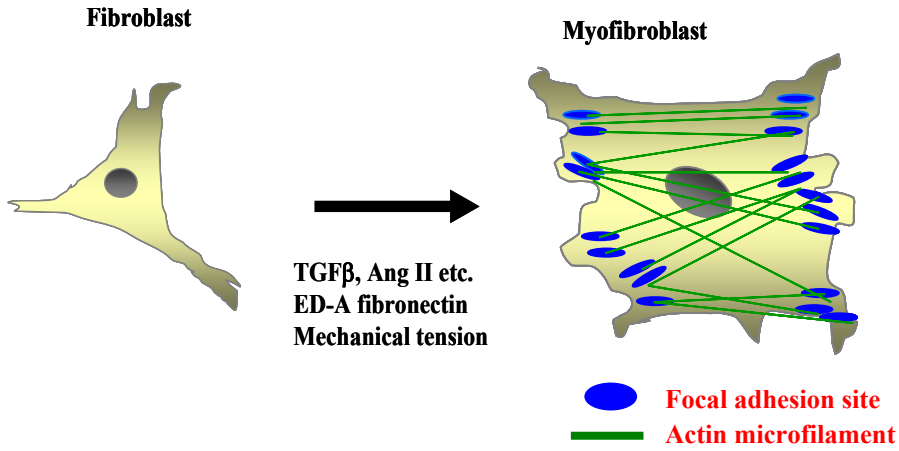


Figure 1-1. Schematic showing the cellular hypertrophy and increased actin microfilament and focal adhesion formation that occurs during fibroblast-myofibroblast transformation.

Fibroblast-to myofibroblast transformation is controlled by a variety of growth factors, cytokines and mechanical stimuli [9, 32, 33]. Among these factors, TGFβ is the most potent stimulator of fibroblast-myofibroblast differentiation and the resulting increases in collagen production [20, 37, 38]. A primary component of fibroblast-to-myofibroblast differentiation is the formation of a contractile apparatus composed of a network of α-SMA-containing microfilaments or stress fibers. Stress fibers anchor and radiate out from distinct regions of the plasma membrane, called focal adhesions, where integrin receptors bind to the ECM [39]. Focal adhesions are sites where anchoring proteins such as vinculin, paxillin, talin and α-actinin link actin cytoskeleton to transmembrane integrin receptors [40].

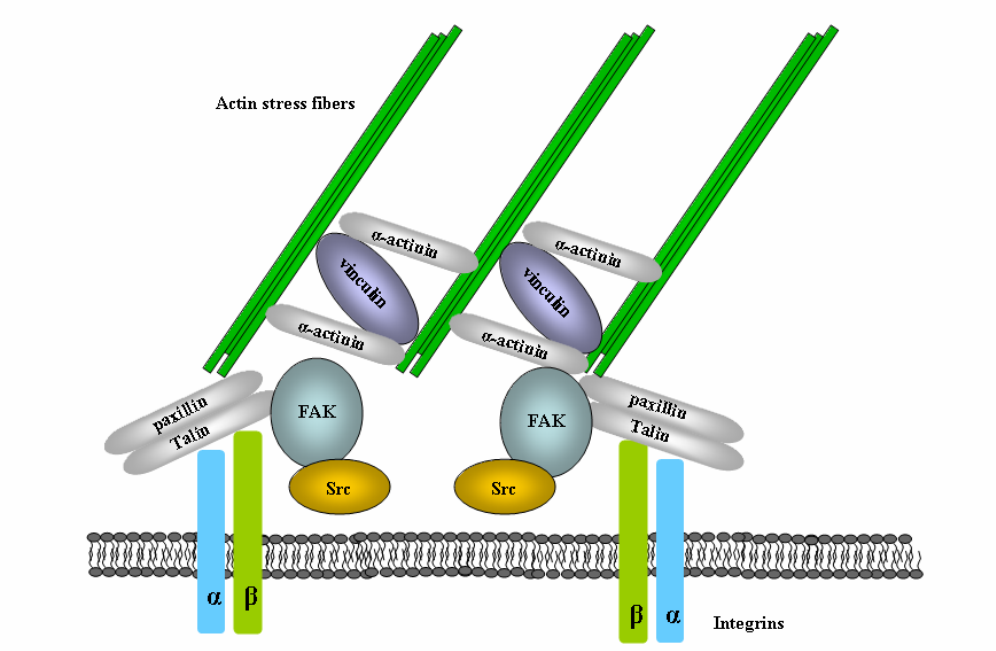


Figure 1-2. Architecture of a focal adhesion showing organization of the structural proteins, actin, α-actinin, vinculin, paxillin and talin and catalytic proteins, focal adhesion kinase (FAK) and src. Modified from Mitra S.K et al. *Nat Rev Mol Cell Biol*. 2005 Jan;6(1):56-68.

Formation of focal contacts involves rapid tyrosine phosphorylation of the non-receptor protein tyrosine kinase, focal adhesion kinase (FAK) [41]. In response to growth factor stimulation and integrin engagement, FAK is autophosphorylated on Tyr397 [42], providing a high affinity binding site for the SH2 domain of src kinase. Binding of src to Tyr397 of FAK permits src-mediated phosphorylation of additional tyrosines residues in the FAK catalytic domain, resulting in full activation of FAK [43]. In addition to providing a binding site for Src tyrosine kinase, phosphorylation at Tyr397 also couples FAK to numerous downstream signaling pathways that regulate cell morphology and behavior [44]. Thus, focal adhesion proteins maintain the

structural integrity of actin/focal adhesion complexes and scaffold catalytic proteins that regulate cell proliferation, survival and motility [41].

Adenylyl cyclases (AC), membrane-bound proteins that catalyze conversion of ATP to cyclic AMP (cAMP), are activated upon stimulation of G protein-coupled receptor (GPCR) agonists that signal though $G_{\alpha S}$. AC's consist of two bundles of six transmembrane segments with two cytoplasmic catalytic domains. Previous studies show that AC is the component in the GPCR-G-protein-AC pathway that limits agonist-mediated increases in maximal cAMP production, including in cardiac myocytes [45-47]. AC6, one of the 9 membrane isoforms of AC, is expressed in many tissues, including CF, and is subject to inhibition by many factors, including $G_{\alpha I}$, $G_{\beta\gamma}$, Ca^{++}, protein kinase C and nitric oxide [48, 49].

regulators of AC activity

AC isoforms	Fsk	$G_{\alpha s}$	$G_{\alpha i}$	$G_{\beta\gamma}$	Ca^{2+}	Ca^{2+}-CaM	PKA	PKC	NO
I	+	+	-			+			
II	+	+		+				+	
III	+	+				+			
IV	+	+		+					
V	+	+	-	-	-		-	+	-
VI	+	+	-	-	-		-	-	-
VII	+	+						+	
VIII	+	+				+			
IX	(+)	+		+					

Figure 1-3. AC isoform-specific regulation by cellular signals. Regulators are identified as activators (+) and inhibitors (-) of AC activation.

Previous studies have shown that AC6 mRNA expression and total AC activity are reduced following pacing-induced heart failure in pigs [50]. In contrast, intracoronary delivery of adenovirus encoding AC6 increases left ventricular (LV) function and attenuates the deleterious remodeling associated with congestive heart failure in pigs [51]. In a mouse model of Gq cardiomyopathy, cardiac-directed expression of AC6 restored myocyte AC function, improved heart function, increased cAMP generation, abrogated myocardial hypertrophy, and increased survival [52, 53]. These studies demonstrate numerous beneficial effects of AC in the heart which result primarily [54] from increased cAMP production in response to AC6 stimulation.

cAMP is a ubiquitous second messenger that influences growth, death, and differentiated functions of many cell types, primarily by promoting protein phosphorylation via cAMP-dependent protein kinase (PKA).

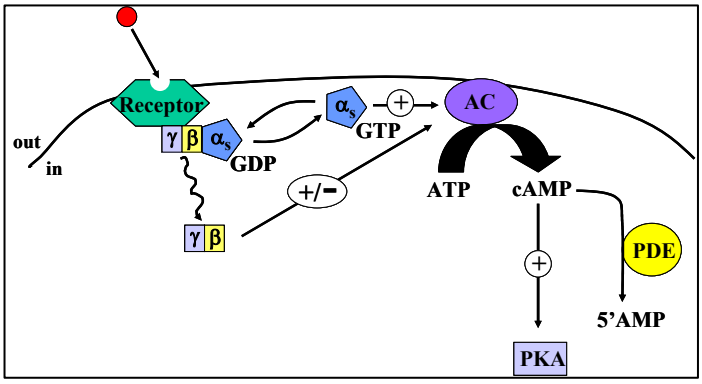


Figure 1-4. Schematic representation of GPCR-mediated activation of adenylyl cyclase (AC). Following ligand binding to GPCR's, the alpha (α) subunit of G_S binds and activates AC. Active AC catalyzes the conversion of ATP to cAMP leading to subsequent activation of protein kinase A (PKA). cAMP is then quickly hydrolyzed to 5' AMP by phosphodiesterases (PDE's).

cAMP levels are regulated by AC activity as well as cyclic nucleotide phosphodiesterases (PDE's) that catalyze its degradation. Several studies have shown that GPCR agonists that activate AC and stimulate cAMP production can inhibit collagen synthesis. Prostaglandin E2 (PGE_2) inhibited collagen type I mRNA expression in human embryo lung fibroblast in a cAMP-dependent manner [55]. Adrenomedullin, an endogenous vasodilator and natriuretic peptide that couples to $G_{\alpha S}$, inhibits neonatal cardiac fibroblast proliferation and collagen production in a cAMP-dependent manner [56]. Furthermore, treatment of cardiac fibroblasts with adenosine inhibited serum-stimulated collagen and total protein synthesis via activation of A2B receptors [57]. Increased cellular levels of cAMP also inhibits TGFβ-induced CTGF gene expression and CTGF-induced collagen production in fibroblasts [58-60]. Recently, the direct AC activator forskolin, and GPCR agonists that stimulate AC were shown to inhibit serum- or TGFβ-stimulated myofibroblast transformation and collagen formation by human lung fibroblasts [61, 62]. FAK phosphorylation at Tyr397 is required for myofibroblast transformation of pulmonary and scleroderma fibroblasts [63, 64]. Via its effects on FAK Y397 phosphorylation, prostaglandin-stimulated cAMP production induces disassembly of actin stress fibers and focal adhesions in aortic smooth muscle cells, with subsequent effects on cell migration [65]. Overall, these findings suggest that AC activation inhibits myofibroblast differentiation and collagen production, in part, via regulation of actin cytoskeleton assembly during fibroblast-to-myofibroblast transformation, which may involve cAMP-mediated regulation of FAK. However, no studies have examined the

effects AC activation on myofibroblast transformation of CF or investigated the role of FAK.

Several AC isoforms and GPCR signaling molecules [66, 67] localize within specialized membrane micro-domains called caveolae. Caveolae are cholesterol and sphingolipid-rich, flask-like invaginations of the plasma membrane [68, 69], that contain the characteristic structural scaffolding proteins, caveolin-1, -2, and -3 [70]. Caveolin-1 and-2 are expressed on adipocytes, endothelial cells, smooth muscle cells and fibroblasts whereas caveolin-3 is muscle-specific [71]. Caveolae act as organizing centers for receptors, signaling components, and effector molecules to facilitate coordinated, precise, and rapid regulation of cell function [72-74].

In addition to their role as scaffolding proteins, caveolins-1 and -2 are substrates for phosphorylation by tyrosine kinases [75, 76]. Previous investigators have shown that only a fraction (~1-5%) of the total cellular caveolin-1 (cav-1) undergoes tyrosine phosphorylation [75, 77]. In response to cellular stress, hormone and growth factor stimulation, cav-1 is phosphorylated on tyrosine 14, located within the N-terminus of the caveolin protein [75, 78, 79]. Once phosphorylated, tyrosine 14 appears to serve as a docking site for SH2 domain-containing proteins such as Grb7 that link cav-1 to other tyrosine phosphorylated proteins such as FAK [75, 80]. Interestingly, phosphorylated cav-1 (phospho-cav-1) localizes primarily along the plasma membrane in close proximity to focal adhesions where actin microfilaments anchor [78]. This suggests that phospho-cav-1 may scaffold catalytic molecules, such

as AC, in close proximity to actin and focal adhesion structural proteins thereby facilitating rapid precise regulation of cell morphology and function.

In this thesis, I hypothesized that AC activation and increased cAMP negatively regulates cardiac fibrosis via effects cardiac fibroblast phenotype and function. More specifically, I hypothesized that cAMP/PKA inhibits cardiac fibrosis by blunting the transformation of cardiac fibroblasts into collagen-producing myofibroblasts. Furthermore, I hypothesized that the cellular distribution of AC5/6, cav-1 and focal adhesion proteins provides a morphologically favorable organization of cellular components to facilitate rapid, coordinated regulation of actin/focal adhesion dynamics by increased cAMP/PKA, resulting in inhibition of fibroblast-to-myofibroblast transformation. To test these hypotheses I carried out specific aims designed to examine:

1. **AC activity and collagen production by CF isolated from rats 18 weeks after myocardial infarction.**
2. **The role of AC activation and overexpression on fibroblast-to-myofibroblast transformation, collagen production and expression of profibrotic proteins (PAI-1 and IL-6) by adult rat CF.**
3. **Whether the interaction between AC, cav-1 and focal adhesion proteins in adult rat CF facilitates cAMP-mediated regulation of fibroblast-to myofibroblast transformation.**

In the course of my studies I have made the following key findings:

- **AC activity and expression are decreased and collagen expression is increased in cardiac fibroblasts isolated 18 weeks after myocardial infarction in rats.**
- **AC activation and a cAMP analog inhibit collagen production in response to multiple pro-fibrotic factors in cardiac fibroblasts.**
- **The inhibition of collagen production in response to AC activation parallels an inhibition of fibroblast-to-myofibroblast transformation.**
- **cAMP production, inhibition of collagen synthesis and inhibition of fibroblast-to-myofibroblast transformation are enhanced by AC agonists in fibroblasts that overexpress AC6.**
- **A portion of AC 5/6 is scaffolded by phosphorylated caveolin-1 at focal adhesion sites, independent of caveolae, in cardiac fibroblasts.**
- **AC activation and a cAMP analog promote caveolin-1 phosphorylation and inactivate focal adhesion kinase.**
- **AC/cAMP-mediated cav-1 phosphorylation and focal adhesion kinase inactivation precede disruption of actin and focal adhesion assembly.**
- **The ability of AC activation to disrupt actin cytoskeleton, stimulate caveolin-1 phosphorylation and inactivate focal adhesion kinase are abrogated by inhibition of the protein tyrosine phosphatase 1B (PTP1B).**

The data outlined in this thesis provide several key findings regarding cardiac fibroblast cell biology and function, specifically as it relates to the role of AC in cardiac fibrosis. These findings show that reduced AC activity may be related to the increased collagen production and subsequent cardiac fibrosis that results from chronic myocardial infarction. The ability of AC/cAMP/PKA to blunt collagen synthesis results from an inhibition of fibroblast-to-myofibroblast transformation and overexpression of AC6 enhances this response. Furthermore, phospho-cav-1-mediated scaffolding of AC at FA sites, independent of caveolae, facilitates the rapid dysregulation of actin and focal adhesion assembly which leads to inhibition of fibroblast-myofibroblast transformation and these effects appear to be dependent on activation of protein tyrosine phosphatase 1B. Thus, these data document a key anti-fibrotic action of AC/cAMP in cardiac fibroblasts and suggest that overexpression of AC6 may provide a novel means to blunt cardiac fibrosis. In addition these studies in this thesis provide new mechanistic information concerning the ability of cAMP/PKA to control multiple downstream catalytic molecules in a manner that inhibits fibroblast-myofibroblast transformation, thereby identifying novel targets for clinical studies aimed at attenuating fibrosis in the heart as well as other organs.

Chapter 2

Adenylyl cyclase activity and function are decreased in rat cardiac fibroblasts after chronic myocardial infarction

Abstract

Myocardial infarction is associated with left ventricular remodeling involving ventricular hypertrophy, dilatation and fibrosis. Cardiac fibrosis, resulting from increased extracellular matrix (ECM) production, contributes to diastolic dysfunction by increasing myocardial stiffening, impairing ventricular filling and thus reducing cardiac output. Adenylyl cyclase (AC) stimulation inhibits ECM production in isolated cardiac fibroblasts and reduced AC function and expression are observed in animal models of heart failure. Here, we examine AC function and the effects of AC stimulation on ECM production using cardiac fibroblasts isolated from adult rats 18 weeks after myocardial infarction (MI). Rats underwent coronary artery ligation or sham surgery (control) and echocardiography was used to assess left ventricular remodeling before and 1, 3, 5, 7, 10, 12 and 18 weeks after surgery. Cardiac fibroblasts isolated from peri-infarct (non-infarcted left ventricular freewall) and distal (septal) regions, remote from the infarct site, were compared for differences in AC activity and collagen synthesis. End-diastolic dimension was increased (Con: 0.8 ± 0.02 (SEM) cm, MI: 1.0 ± 0.02 cm; $p<0.001$, $n\geq5$) and fractional shortening decreased (Con: 44 ± 2%, MI: 20 ± 2%; $p<0.001$, $n\geq5$). AC activity in cardiac fibroblasts (passage ≤ 2) from peri-infarct and distal regions of the left ventricle revealed a 66% and 62% decrease ($p<0.05$, $n\geq3$), respectively, in forskolin-stimulated cAMP production, compared to shams. Serum-stimulated collagen production was increased 2.0 and 3.2 fold ($p<0.001$, $n\geq3$) in peri-infarct and distal regions respectively, compared to shams. Overall, these data demonstrate that AC activity and expression

are reduced and collagen production is increased in cardiac fibroblasts isolated from rats after MI. Furthermore, these data suggest that the *in vivo* sequelae of heart failure can be studied using early passage cells isolated from rats after MI and that reduced AC activity may be related to the increased collagen production and cardiac fibrosis resulting from chronic myocardial infarction.

Introduction

Congestive heart failure (CHF) is a leading cause of morbidity and mortality in the U.S. and currently affects nearly 5 million people. Initially, CHF was believed to result primarily from systolic dysfunction, or more specifically, an inability of the heart to pump blood. More recently, it is recognized that diastolic dysfunction, or an inability of the heart to fill with blood during diastole, is the underlying mechanism in a large proportion of patients with heart failure [2]. Myocardial fibrosis, a key contributor to diastolic dysfunction during CHF, reflects hyperplasia and increased deposition of extracellular matrix (ECM) material into the interstitial and perivascular space [4]. Exaggerated ECM deposition results in myocardial stiffening and decreased relaxation of the heart, ultimately leading to diastolic dysfunction and heart failure [3, 6].

The normal myocardium is composed of cardiac myocytes and non-myocytes, which include endothelial cells, vascular smooth muscle cells and fibroblasts [7]. Cardiac fibroblasts (CF), the most abundant cell type in the heart (comprising ~ 2/3 of the total cell population) are responsible for basal ECM homeostasis as well repair after cardiac insult [3, 8]. Following myocardial infarction (MI), reparative scar

formation at the site of injury is often accompanied by maladaptive connective tissue production, remote from the infarct zone, in the remaining, functional regions of the myocardium [10-12]. This excess scar tissue impairs cardiac compliance and reduces filling capacity, ultimately resulting in diastolic heart failure.

Adenylyl cyclases (AC), membrane-bound proteins that catalyze conversion of ATP to cyclic AMP (cAMP), are activated upon stimulation of G protein-coupled receptor (GPCR) agonists that signal though $G_{\alpha S}$. Of the nine membrane-bound AC isoforms, adenylyl cyclase type 5 (AC5) and type 6 (AC6) are the most highly expressed isoforms in the heart and have direct implications on cardiac function. [48, 49, 81]. AC6 mRNA expression and total AC activity are reduced in animal models of heart failure [50] and overexpression of AC6 attenuates deleterious remodeling and increases function in the heart [51-53]. Our lab and others have shown that AC activation and increased cAMP production can inhibit fibroblast proliferation and collagen synthesis, suggesting an anti-fibrotic role for AC in the heart [55-57, 67, 82]. Although these data demonstrate an anti-fibrotic role for AC in the heart, the relationship between AC function and collagen production by CF after MI is not known.

We hypothesized that down regulation of AC in the heart following MI may contribute to increased collagen production by CF resulting in myocardial stiffening and diastolic dysfunction. To test this hypothesis we conducted echocardiographic assessment of left ventricular (LV) function over an 18 week period after myocardial infarction and analyzed changes in AC function and collagen production by adult rat

CF isolated at 18 weeks post-MI. Using CF isolated from different regions of the rat LV, we show that total AC activity and AC5/6 expression are reduced concomitant with increased collagen production. These data suggest that decreased AC function in the heart after MI may contribute to maladaptive connective tissue production and subsequent diastolic heart failure.

Materials and Methods

Animals

Animals were handled in accordance with the animal welfare regulations of the VA San Diego Healthcare System and NIH guidelines. Adult male (200 – 250 g) Sprague Dawley rats were used. Twenty-five rats underwent thoracotomy and coronary artery ligation (MI group) and 8 rats underwent thoracotomy and no coronary artery ligation (control group). All animals were killed 18 weeks after surgery.

Myocardial Infarction

Anesthetic induction was accomplished using isoflurane (5% in 100% O_2). The rats were inverted and the trachea and vocal chords were visualized to enable placement of an endotracheal guide wire. Rats were then intubated using a 16 gauge angiocatheter and mechanically ventilated with 1.5% isoflurane in 100% O_2 to maintain anesthesia during surgery. Animals then were placed in the left lateral decubitis position and the chest cavity was opened at the third intercostal to visualize

the left side of the heart. The left coronary artery was ligated approximately 2 mm below the edge of the left atrial appendage by placing 7-0 prolene suture (tapered needle) around the artery. LV blanching indicated successful occlusion of the vessel. The chest was then closed by suturing together adjacent ribs (6-0 nylon), and the skin closed as a separate layer.

Echocardiography

Echocardiography was performed in anesthetized rats prior to surgery and at 1, 3, 5, 7, 10, 12, 14 and 18 weeks post-surgery. Rats were anesthetized using isoflurane anesthesia (5% in 100% O_2) supplied via a nose mask. Using a pediatric 12 MHz linear probe (Agilent Technologies) a parasternal short axis view was obtained as a guide for LV M-mode imaging at the papillary muscle level. The M-mode images were digitized on the optical disc (HP 5500). Using the HP 5500 standard software, LV dimensions were traced in both end-diastole and end-systole in short and long axis views and LV volumes determined using the modified Simpson method and percentage fractional area change (%FS) was calculated. Infarct size was assessed by measuring regional wall motion in both long axis and short axis modes and assigning an echo score based on those measurements. An infarct was considered to be small if the echo score was between zero and three and large if it was four or greater [range of echo score is 0 (no infarct) to 8 (large infarct)]. Only animals with an echo score ≥ 4 were included in the infarct group.

Isolation and Culture of Adult Rat CF

Eighteen weeks after MI, cardiac fibroblasts were isolated from the peri-infarct (non-infarcted LV freewall) region and distal (LV septum) regions using a modification of published methods [83]. Briefly, animals were heparinized (1000-2000 units IP) 5 min prior to being anesthetized with ketamine (100mg/kg) and xylazine (10mg/kg); hearts were then removed and placed in ice-cold cardioplegic (20 mM KCl) solution (in mmol/L: 112 NaCl, 5.4 KCl, 1 $MgCl_2$, 9 NaH_2PO_4. 11.1 D-glucose; supplemented with 10 Hepes, 30 Taurine, 2 DL-carnitine, 2 creatine, pH. 7.4). The hearts were retrograde-perfused at 37°C on a Langendorff apparatus with Ca^{2+}-free heart media for 5 min at 5 ml/min, followed by perfusion with a Ca^{2+}-free heart media containing collagenase II (Worthington Biochemical Corp., cat# CLS 2) for 20 min. The right ventricles (RV) were removed and the LV's were separated in to peri-infarct and distal regions. Individual LV regions were minced in collagenase II-containing medium for 10-15 min. CF were separated from cardiac myocytes by gravity separation and grown to confluency on 10 cm cell culture dishes at 37^oC with 90% air/10% CO_2 in growth media (DMEM/10% fetal bovine serum [FBS]/1% penicillin/1% streptomycin). All CF were used at early passage (pass ≤ 2) to minimize loss of the *in vivo* phenotype as a result of cell division i*n vitro*.

Collagen synthesis assay

We assessed collagenase-sensitive [^{3}H]-proline incorporation (using a modification of established methods [84]) to measure collagen synthesis. CF cultured

on 12-well plates were serum-starved for 48 hr and then assayed by adding 1 μCi/ml of [^{3}H]-proline (Perkin Elmer Life Sciences) along with drugs of interest and, where indicated, 0% or 2.5% FBS for 48 hr. Cells were removed from tissue culture dishes with trypsin and protein was precipitated overnight with 20% trichloroacetic acid (TCA). Following centrifugation, pellets were washed 3 times with 1.0 ml 5% TCA + 0.01% proline, then dissolved with 0.2 M NaOH and the solutions titrated to neutral pH with 0.2 M HCl. Collagenase II (100 μl, 2 mg/ml) in Tris-$CaCl_2$-N-ethylmaleimide buffer was added to each tube and samples were incubated for 1 hr at 37 ^oC. Samples were then placed on ice and proteins precipitated with 10% TCA for 1 hr, centrifuged at 18, 000 x g and radioactivity of the supernatant fraction was determined by liquid scintillation counting.

Cyclic AMP production

CF grown on 24-well plates were serum-starved for 48 hr and assayed for cAMP production by 10 min incubation with 0.2 mM isobutylmethylxanthine (IBMX), a cyclic nucleotide phosphodiesterase inhibitor, followed by addition of drugs of interest for an additional 10 min. Assays were terminated by aspiration of media and addition of 250 μl of ice-cold TCA (7.5%) to each well. Radioimmunoassay was used to quantitate cAMP [62, 67]; results were normalized to the amount of protein per sample, as determined by a colorimetric protein assay (Bio-Rad).

Immunoblot Analysis

Antibodies for AC type 5/6 were obtained from Santa Cruz. CF whole cell lysates were prepared in cell lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, protease inhibitor cocktail) and homogenized by sonication. Equal protein (assayed using a dye-binding reagent [Bio-rad]) amounts of the lysates were separated by SDS-polyacrylamide gel electrophoresis using 10% polyacrylamide precast gels (Invitrogen) and transferred to a polyvinylidene difluoride (PVDF) membrane by electroblotting. Membranes were blocked in 20 mM PBS Tween (1%) containing 1.5% nonfat dry milk and incubated with primary antibody overnight at 4°C. Bound antibodies were visualized using secondary antibodies with conjugated horseradish peroxidase (Santa Cruz Biotechnology) and ECL reagent (Amersham Pharmacia Biotech). All displayed bands were compared to molecular weight standards to confirm that the proteins migrated at the appropriate size.

Data analysis

Statistical comparisons and graphical representation were performed using Graph Pad Prism 3.0 (GraphPad Software). Statistical significance was set at $p<0.05$.

Results

Echocardiographic assessment of LV size and function following MI

Compared to control animals, rats with MI exhibited increased LV chamber diameter and dramatic thinning of the anterior LV freewall, indicative of myocardial

tissue loss in the infarct region (Fig 2-1A and B). LV remodeling post-MI was associated with a significant ($p<0.05$) decrease in % fractional shortening (%FS; Fig 2-1C) and increased end-diastolic dimension (EDD; Fig 2-1D), demonstrating reduced cardiac output and progressive LV chamber dilatation following MI. No significant changes in %FS or EDD were observed in control animals. Thus, cardiac remodeling was increased and function was impaired in MI rats compared to controls.

AC activity of CF is reduced following MI

In order to analyze differences in AC activity by CF isolated post-MI, we measured cAMP production under basal conditions and in response to the direct AC agoinst, forskolin (fsk; 10 μM), using CF isolated from peri-infarct and distal region of the rat heart. Although there were no differences in basal cAMP production, there was a significant ($p<0.05$) decrease (Fig 2-2A and B) in fsk-stimulated cAMP production by CF from MI rats compared to controls. We observed a similar reduction in fsk-stimulated cAMP production by isolated cardiac myocytes demonstrating that, after MI, AC activity is reduced in multiple cardiac cell types (data not shown). No statistically significant regional differences in cAMP production were observed in CF prepared from peri-infarct and distal zones of either control or infarct rats. Although there were no regional differences in AC activity from either infarct or control animals, the results show that agonist-stimulated AC activity is reduced in CF isolated from rats post-MI, compared to controls.

AC expression is reduced following myocardial infarction

In order to determine whether decreased cAMP production by CF results from reduced AC expression, we examined expression of AC 5/6, the predominant AC isoforms expressed in the heart [48, 49, 81], using immunoblot. We found that AC 5/6 expression was significantly ($p<0.05$) reduced in CF from infarcted rats compared to controls, however there were no regional differences in AC 5/6 expression in CF isolated from peri-infarct or distal zones (Fig 2-3A and B). Thus, decreased AC activity by CF following MI appears to result from reduced myocardial AC expression.

Collagen production by CF is increased following MI

Because AC activation exerts anti-fibrotic effects on fibroblasts in culture [55-57, 67, 82], we hypothesized that the decreased AC expression and activity may correlate with increased collagen production by CF following MI. Consistent with this hypothesis, CF from infarcted rats exhibited enhanced ($p<0.05$) serum-stimulated collagen production (as indicated by [^{3}H]-proline incorporation) compared the control CF isolated from both peri-infarct and septal regions (Fig. 2-4A and B). Serum-stimulated collagen production from both control and infarct rats was reduced ($p<0.05$) by AC stimulation with forskolin (fsk; 10 μM), demonstrating the anti-fibrotic effects of AC activation in isolated CF. Although forskolin reduced serum-stimulated collagen production down to basal levels in control CF, CF from infarcted rats exhibited only moderate inhibition in response to forskolin treatment. These data

are consistent with the decreased AC activity (Fig 2-2A and B) and expression (Fig 2-3A and B) by CF from infarcted rats and suggest that increased collagen production by CF following MI results from decreased AC expression and a decreased ability of CF to produce cAMP.

Discussion

In this study we sought out to determine whether the fibrosis and maladaptive connective tissue production that leads to diastolic dysfunction late after MI results, at least in part, from a downregulation of AC following myocardial insult. Previous studies have demonstrated cardioprotective effects of AC using both *in vivo* and *in vitro* models and AC expression level has been correlated with differences in cardiac function [48, 49, 81]. AC6 mRNA expression and total AC activity are reduced following pacing-induced heart failure in pigs [50]. In contrast, intracoronary delivery of adenovirus encoding AC6 increased left ventricular (LV) function and attenuated the deleterious remodeling associated with CHF [51]. In a mouse model of Gq cardiomyopathy, cardiac-directed expression of AC6 restored myocyte AC function, improved heart function, increased cAMP generation, abrogated myocardial hypertrophy, and increased survival [52, 53]. These findings demonstrate numerous beneficial effects of AC in the heart which are believed to result from increased cAMP production in response to AC6 stimulation. Although these studies demonstrate the ability of AC to maintain function and attenuate remodeling of the muscle portion of the heart, they failed to examine the effects of AC on the predominant non-muscle cells in the heart (ie., cardiac fibroblasts) that produce exaggerated amounts of

collagen in the non-infarcted myocardium after MI, leading to myocardial stiffening and diastolic dysfunction.

Previous *in vitro* studies have demonstrated anti-fibrotic effects of AC activation, whereby G protein-coupled receptor (GPCR) agonists that activate AC and stimulate cAMP production can inhibit collagen synthesis by fibroblasts in culture [55-57, 67] [61, 62]. Recent data from our lab has shown that the anti-fibrotic effects of AC are enhanced by cardiac fibroblasts overexpressing AC6. Together, these data suggest that AC may exhibit anti-fibrotic effects in the heart following MI. However, to date, no studies have examined AC function and collagen production by cardiac fibroblasts isolated from different region of the heart to assess the role of AC in connective tissue remodeling late after MI. In this study we provide the first evidence demonstrating long-term (18 week) decreases in AC activity and expression by CF isolated from rat hearts following MI. Furthermore, we show that decreased AC function correlates with increased collagen production by CF. Thus, the cardiac fibrosis following MI may result, in part, from a downregulation of AC thereby reducing the ability of AC to negatively regulate the exaggerated connective tissue production by CF after MI.

One limitation of the current study is the potential loss of the pro-fibrotic phenotype by CF that are removed from the *in vivo* cardiac milieu and studied under *in vitro* culture conditions. For this reason, we conducted all experiments using very low passage cells (pass ≤ 2) to avoid any de-differentiation of the CF resulting in a change in the heart failure phenotype. The methodologies presented here are consistent with

previous reports examining protein expression and signaling by CF isolated rats after MI and studied in culture[85-88]. Unlike these studies which examined CF at early timepoints (≤ 4 weeks) post-MI, we examined the long term effects of MI on AC function and collagen production to understand the role of AC in the late phase remodeling that leads diastolic dysfunction. The progressive fibrosis in the non-infarcted myocardium that leads to diastolic dysfunction in mice occurs much later, reaching a maximum at approximately 4 months post-MI [89].

Therefore, these data provide beneficial new information regarding AC expression and activity levels by CF following myocardial insult and its potential role as an anti-fibrotic mediator of the deleterious connective tissue remodeling that occurs late after MI. Furthermore these finding are the first to demonstrate that in vitro techniques can be used to study the long term sequelae of diastolic heart failure using to study isolated CF after myocardial infarction in rats.

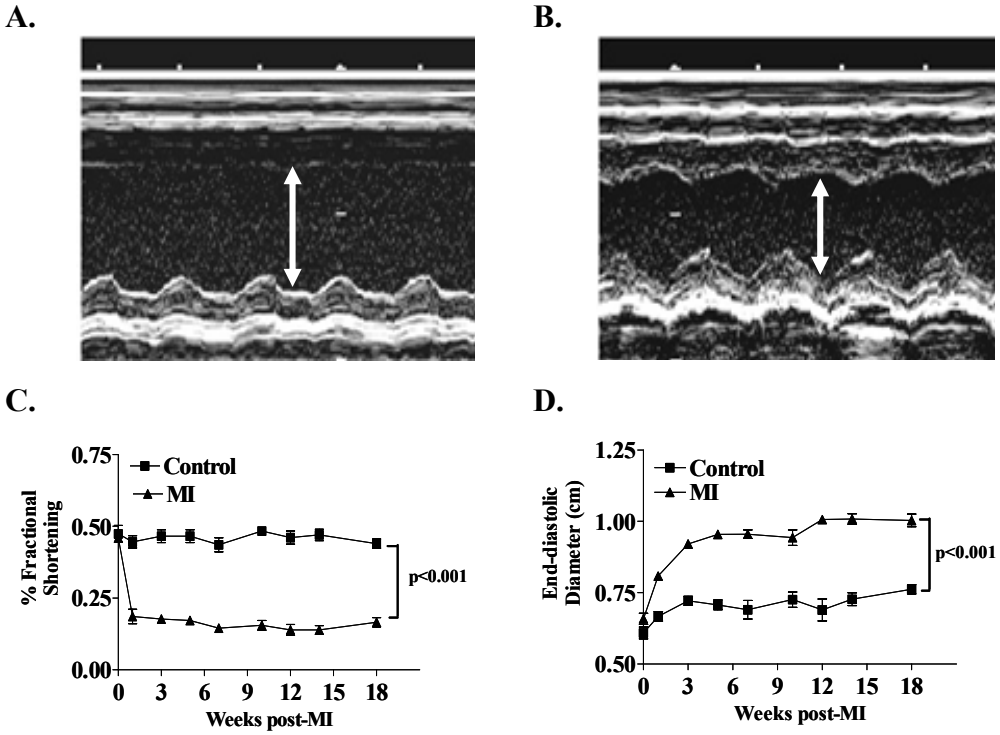


Figure 2-1. Cardiac size is increased and cardiac function is reduced in adult rat CF 18 weeks after myocardial infarction. M-mode echocardiographic images were obtained after infarct (MI) or sham surgery (control). Arrows denote the distance between the anterior LV freewall and the posterior LV (septum) during diastole. (C) % fractional shortening (%FS) and (B) end-diastolic diameter (EDD) were measured before and 1, 3, 5, 7, 10, 12, 14, and 18 weeks after myocardial infarction or sham-surgery to assess LV function and dilatation. Values represent mean ± SEM of at least 5 experiments compared using Two-way analysis of variance with post-hoc multiple comparisons test. $p<0.05$ denotes control vs. MI rats.

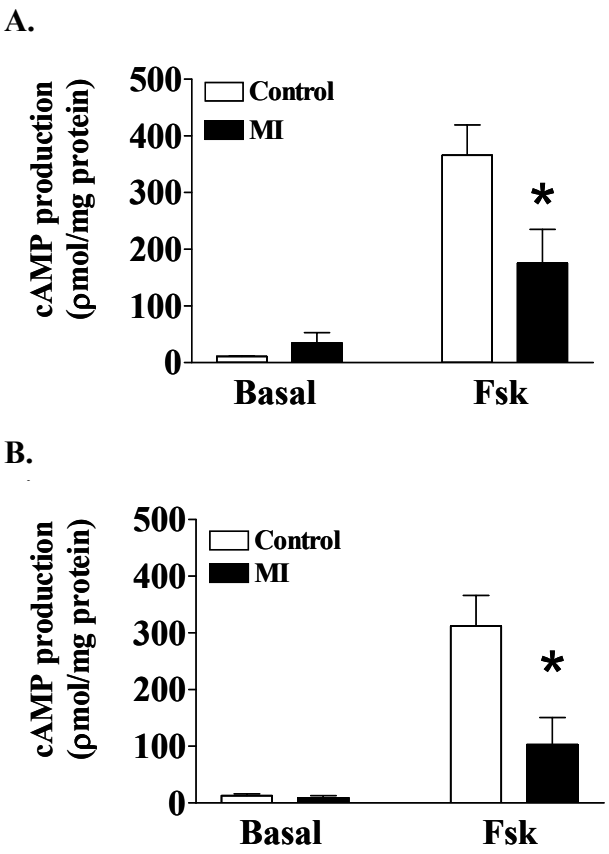


Figure 2-2. cAMP production is reduced in adult rat CF 18 weeks after myocardial infarction. cAMP production by CF isolated from (A) septum and (B) peri-infarct regions of rats 18 weeks after infarct (MI) or sham surgery (control). cAMP production was measured by radioimmunoassay using CF grown for 48 hr in serum-free media and then stimulated for 10 min with 2.5% fetal bovine serum (FBS) alone (basal) or in the presence of forskolin (Fsk, 10 μM). Values represent mean ± SEM of at least 3 experiments and compared using a Two-way analysis of variance with post-hoc multiple comparisons test. * denotes $p<0.05$ compared to control.

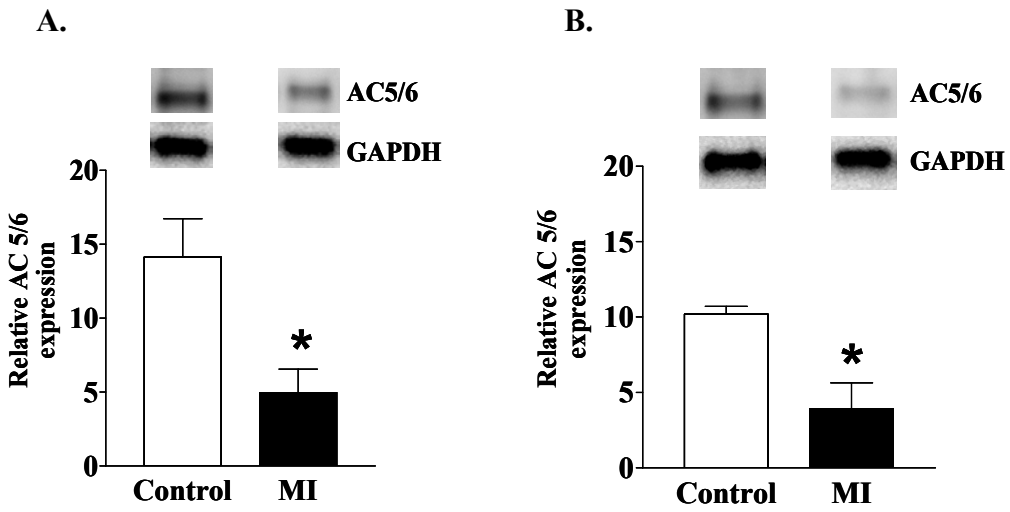
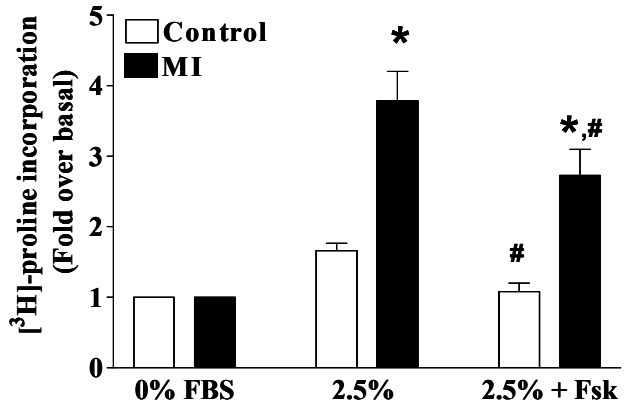


Figure 2-3. AC5/6 expression is reduced in adult rat CF 18 weeks after myocardial infarction. AC 5/6 expression was measured by immunoblot using CF (passage ≤2) isolated from (A) septum and (B) peri-infarct regions of rats 18 weeks after infarct (MI) or sham surgery (control). Representative AC 5/6 and GAPDH (loading control) immunoreactive bands are shown for comparison. AC5/6 expression values were normalized to GAPDH expression and data are expressed as relative AC 5/6 expression. Values represent mean ± SEM of at least 3 experiments and compared using an unpaired Student's t-test. * denotes $p<0.05$ compared to control.

A.



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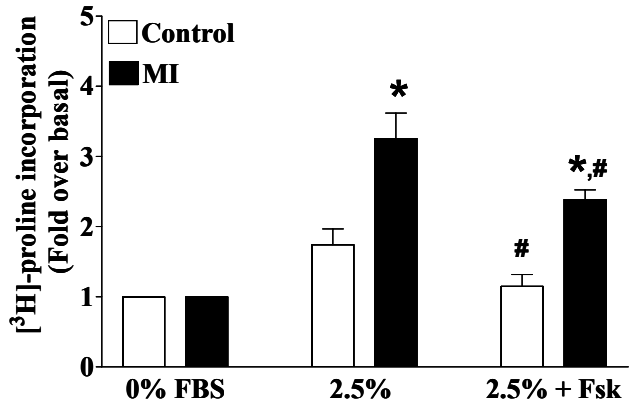


Figure 2-4. Collagen production is enhance in adult rat CF 18 weeks after myocardial infarction. Collagenase sensitive [3H]-proline incorporation by CF isolated from (A) septum and (B) peri-infarct regions of rats 18 weeks after MI (Infarct) or sham surgery (control). CF were grown for 48 hr in serum-free media and then stimulated for 48 hr with 0% fetal bovine serum (FBS) or 2.5% in the absence or presence of forskolin (Fsk, 10 μM). Data are normalized for [^{3}H]-proline incorporation into cells grown under 0% FBS conditions. Values represent mean ± SEM of at least 3 experiments and compared using a Two-way analysis of variance with post-hoc multiple comparisons test. * denotes $p<0.05$ compared to control. # denotes $p<0.05$ compared to 2.5% FBS.

Chapter 3

Inhibition of cardiac myofibroblast formation and collagen synthesis by activation and over-expression of adenylyl cyclase

Abstract

Transformation of fibroblasts to myofibroblasts, characterized by expression of α-smooth muscle actin (α-SMA) and production of extracellular matrix (ECM) components, is a key event in connective tissue remodeling. Approaches to inhibit this transformation are needed in tissues, such as the heart, where excessive ECM production by cardiac fibroblasts (CF) causes fibrosis, myocardial stiffening and cardiac dysfunction. We tested whether adenylyl cyclase (AC) activation (increased cAMP levels) modulates the transformation of adult rat CF to myofibroblasts, as assessed by immunofluorescent microscopy, immunoblotting and collagen synthesis. A 24-hour incubation of CF with TGFβ or angiotensin II increased α-SMA expression, which was inhibited by the AC agonist forskolin and a cAMP analog that activates protein kinase A. Treatment with forskolin blunted serum-, TGFβ- and angiotensin II-stimulated collagen synthesis. CF engineered to over-express type 6 AC had enhanced forskolin-promoted cAMP formation, greater inhibition by forskolin of TGFβ-stimulated α-SMA expression, and a decrease in the EC_{50} of forskolin to reduce serum-stimulated collagen synthesis. The AC stimulatory agonist adrenomedullin inhibited collagen synthesis in CF that overexpressed AC6 but not in controls. Thus, AC stimulation blunts collagen synthesis and, in parallel, the transformation of adult rat CF to myofibroblasts. AC-overexpression enhances these effects, "uncovering" an inhibition by adrenomedullin. These findings implicate cAMP as an inhibitor of ECM formation via blockade of the transformation of CF to myofibroblasts and suggest that increasing AC expression, thereby enhancing cAMP generation through stimulation of

receptors expressed on CF, could provide a novel means to attenuate and prevent cardiac fibrosis and its sequelae.

Introduction

Fibroblasts are widely recognized as a critical cell type involved in wound healing and tissue repair. Less generally appreciated is the notion that the transformation of fibroblasts to myofibroblasts is a key, perhaps essential, event for the cells to perform those functions [3, 9, 32, 90]. Myofibroblasts are smooth muscle-like fibroblasts that express alpha-smooth muscle actin (α-SMA) and contain a contractile apparatus composed of actin filaments and associated proteins organized into prominent stress fibers [9, 91]. In addition to their normal role in tissue homeostasis and repair, altered number and function of myofibroblasts have been implicated in diseases with increased extracellular matrix (ECM) deposition and resultant fibrosis, such as those involving liver, skin, lung, and kidney [34-36].

Myofibroblast formation is controlled by a variety of growth factors, cytokines and mechanical stimuli [9, 32, 33]. Transforming growth factor-β_1 (TGFβ_1) is one such factor that stimulates both myofibroblast formation and collagen production [9, 92]. Such results imply that agents able to inhibit fibroblast-to-myofibroblast transformation may provide a means to inhibit maladaptive tissue remodeling in response to pro-fibrotic stimuli.

Cardiac fibroblasts (CF), the most abundant cell type in the heart (constituting two-thirds of the total cell population), are responsible for ECM deposition and create

the scaffold for cardiac myocytes [8]. Cardiac fibrosis is characterized by overproduction of ECM, predominantly collagen types I and III, into the interstitial and perivascular space [4]. Excessive collagen deposition leads to myocardial stiffening, impaired cardiac relaxation and filling (diastolic dysfunction) and overload of the heart, perhaps as a consequence of transformation of quiescent fibroblasts, responsible for basal ECM homeostasis, to activated myofibroblasts [3].

Cyclic AMP (cAMP), a ubiquitous second messenger produced in response to activation of adenylyl cyclase (AC), influences growth, death, and differentiated functions of many cell types, primarily by promoting protein phosphorylation via cAMP-dependent protein kinase (PKA). G protein-coupled receptor (GPCR) agonists that signal through G_s to activate AC and stimulate cAMP production can inhibit collagen synthesis [56, 57, 67]. Forskolin and GPCR agonists that stimulate AC were recently shown to inhibit serum or TGFβ-stimulated α-SMA expression and collagen formation by human lung fibroblasts [61, 62]. Although such data suggest that increased cAMP production inhibits collagen synthesis via an inhibition of fibroblast-to-myofibroblast transformation, no direct evidence has linked the two events and no studies have examined the effects of increased cAMP on myofibroblast formation in the heart.

We hypothesized that the inhibition of collagen synthesis by cAMP results from its ability to blunt myofibroblast formation. Using both pharmacological activators and gene transfer of AC, we show here that AC activation inhibits myofibroblast formation and in parallel reduces collagen synthesis by adult rat CF,

effects that are strikingly enhanced in cells that overexpress an AC isoform. These results document a key anti-fibrotic action of cAMP in adult CF and suggest that overexpression of AC6 may provide a novel means to blunt cardiac fibrosis.

Materials

Antibodies for α-SMA and vimentin were obtained from Sigma. Antibodies for collagen type I was obtained from Rockland. Alexa Fluor 647 Phalloidin probe for F-actin was obtained from Molecular Probes. Radiolabeled chemicals were obtained from Perkin Elmer. All other drugs and reagents were obtained from Sigma.

Isolation and Culture of Adult Rat CF

CF were isolated from adult Sprague-Dawley rats (250-300 g, male) using a modification of published methods [83]. Briefly, animals were heparinized (1000-2000 units IP) 5 min prior to being anesthetized with ketamine (100mg/kg) and xylazine (10mg/kg); hearts were then removed and placed in ice-cold cardioplegic (20 mM KCl) solution (in mmol/L: 112 NaCl, 5.4 KCl, 1 $MgCl_2$, 9 NaH_2PO_4. 11.1 D-glucose; supplemented with 10 Hepes, 30 Taurine, 2 DL-carnitine, 2 creatine, pH. 7.4). The hearts were retrograde-perfused at 37°C on a Langendorff apparatus with Ca^{2+}-free heart media for 5 min at 5 ml/min, followed by perfusion with a Ca^{2+}-free heart media containing collagenase II (Worthington Biochemical Corp., cat# CLS 2) for 20 min. Both ventricles were then minced and incubated in collagenase II-containing medium for 10-15 min. CF were separated from cardiac myocytes by

gravity separation and grown to confluency on 10 cm cell culture dishes at 37^{o}C with 90% air/10% CO_2 in growth media (DMEM/10% fetal bovine serum [FBS]/1% penicillin/1% streptomycin). For overexpression studies CF were incubated with approximately 100 viral particles per cell of an adenovirus containing either *lacZ* (control) or the murine AC6 gene as described previously [47, 62]. Cells were incubated with adenovirus for 18-24 hr prior to stimulation with serum or agonists of interest. Experiments using LacZ adenovirus and ß-galactosidase staining indicated that the efficiency of the adenoviral gene transfer was greater than 80% (data not shown).

Collagen synthesis assay

We assessed collagenase-sensitive [^{3}H]-proline incorporation (using a modification of established methods [84]) to measure collagen synthesis. CF cultured on 12-well plates were serum-starved for 48 hr and then assayed by adding 1 μCi/ml of [^{3}H]-proline (Perkin Elmer Life Sciences) along with drugs of interest and, where indicated, 0% or 2.5% FBS for 48 hr. Cells were removed from tissue culture dishes with trypsin and protein was precipitated overnight with 20% trichloroacetic acid (TCA). Following centrifugation, pellets were washed 3 times with 1.0 ml 5% TCA + 0.01% proline, then dissolved with 0.2 M NaOH and the solutions titrated to neutral pH with 0.2 M HCl. Collagenase II (100 μl, 2 mg/ml) in Tris-$CaCl_2$-N-ethylmaleimide buffer was added to each tube and samples were incubated for 1 hr at 37 ^{o}C. Samples were then placed on ice and proteins precipitated with 10% TCA for 1 hr, centrifuged

at 14,000 rpm for 10 min and radioactivity of the supernatant fraction was determined by liquid scintillation counting.

Immunoblot Analysis

Whole cell lysates were prepared in cell lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, protease inhibitor cocktail) and homogenized by sonication. Equal protein (assayed using a dye-binding reagent [Bio-rad]) amounts of the lysates were separated by SDS-polyacrylamide gel electrophoresis using 10% polyacrylamide precast gels (Invitrogen) and transferred to a polyvinylidene difluoride (PVDF) membrane by electroblotting. Membranes were blocked in 20 mM PBS Tween (1%) containing 1.5% nonfat dry milk and incubated with primary antibody overnight at 4°C. Bound antibodies were visualized using secondary antibodies with conjugated horseradish peroxidase (Santa Cruz Biotechnology) and ECL reagent (Amersham Pharmacia Biotech). All displayed bands were compared to molecular weight standards to confirm that the proteins migrated at the appropriate size. Antibody specificity was confirmed by positive and negative expression of α-SMA by rat aortic smooth muscle cell and rat neuroepithelial cell lysates, respectively (data not shown).

Immunohistochemistry

CF cultured on 12mm glass coverslips were serum-starved for 48 hr and then stimulated for 24 hr in media alone (control) or with drugs of interest. CF were then

fixed in 10% buffered formalin for 10 min, washed twice with phosphate buffered saline (PBS), and permeabilized in 0.3% Triton X 100/PBS for 10 min. CF were washed twice with PBS/Tween 20 (0.1% Tween), incubated with 1% BSA/PBS/Tween for 10 min and then with an FITC-conjugated α-SMA antibody (1:1000) for 1-2 hr. Following 2 washes with PBS/Tween and incubation with DAPI (1:5000) for 20 min, CF were washed for 10 min with PBS and mounted in gelvatol for microscopic imaging. All incubation and wash steps were conducted at room temperature.

Image Deconvolution analysis

Deconvolution images were obtained as previously described [93]. Images were captured with a DeltaVision deconvolution microscope system (Applied Precision, Inc., Issaquah, WA.) The system includes a Photometrics CCD mounted on a Nikon TE-200 inverted epi-fluorescence microscope. In general, between 30 and 80 optical sections spaced by~ 0.1-0.3 um were taken. Exposure times were set such that the camera response was in the linear range for each fluorophore. Lenses included 100x (NA 1.4), 60x (NA 1.4) and 40x (NA 1.3). The data sets were deconvolved and analyzed using SoftWorx software (Applied Precision, Inc) on a Silicon Graphics Octane workstation.

Cyclic AMP production

CF grown on 24-well plates were serum-starved for 48 hr and assayed for cAMP production by 10 min incubation with 0.2 mM isobutylmethylxanthine (IBMX), a cyclic nucleotide phosphodiesterase inhibitor, followed by addition of drugs of interest for an additional 10 min. Assays were terminated by aspiration of media and addition of 250 μl of ice-cold TCA (7.5%) to each well. Radioimmunoassay was used to quantitate cAMP [62, 67]; results were normalized to the amount of protein per sample, as determined by a colorimetric protein assay (Bio-Rad).

Data analysis

Statistical comparisons and graphical representation were performed using Graph Pad Prism 3.0 (GraphPad Software). Statistical significance was set at $p<0.05$.

Results

Adenylyl cyclase activation inhibits total collagen synthesis and expression of collagen I protein

Using a collagenase-sensitive [^{3}H]-proline incorporation assay as a measure of collagen synthesis, we investigated the effects of forskolin or adrenomedullin (two activators of AC in CF [56, 67]), CPT-cAMP (a cAMP analog which is selective for PKA activation [94]) or CPT-Me-cAMP (a PKA-independent cAMP analog [95]) on serum-stimulated collagen synthesis by adult rat CF. Forskolin (10 μM) or CPT-

cAMP (100 μM), but not adrenomedullin (1 μM) or CPT-Me-cAMP, prominently inhibited 2.5% FBS-stimulated collagen synthesis (Figure 3-1A). Since serum contains multiple hormones and growth factors that might promote collagen synthesis, we examined the effects of forskolin in response to two key pro-fibrotic agents: angiotensin II (Ang II) and transforming growth factor-β (TGFβ), both tested under serum-free conditions [38, 90]. Forskolin significantly ($p<0.05$) inhibited collagen synthesis in response to both these pro-fibrotic agents, as well as to aldosterone (data not shown), thus showing that an increase in cAMP inhibits collagen formation in response to multiple agents that promote fibrosis (Figure 3-1A and B). The results with CPT-cAMP vs. those with CPT-Me-cAMP imply that this effect occurs via PKA activation. To determine the effects of AC activation on formation of a specific collagen found in the heart, we measured expression of collagen type I. Similar to results of collagenase-sensitive [^{3}H]-proline incorporation (Figure 3-1A and B), forskolin inhibited Ang II- and TGFβ-stimulated collagen type I expression (Figure 3-1C). Taken together, these data demonstrate that increases in cAMP act via PKA to inhibit collagen production of adult rat CF stimulated by serum or pro-fibrotic agents.

Phosphodiesterase inhibition blunts spontaneous myofibroblast differentiation and forskolin attenuates TGFβ-stimulated myofibroblast transformation of adult rat CF

Cell culture conditions have been shown to be critical when conducting *in vitro* studies of fibroblast-to-myofibroblast differentiation. Previous studies have shown that fibroblasts rapidly differentiate into myofibroblasts (as indicated by increased α-SMA

expression) when cultured on rigid substrates and/or in culture media containing serum and when plated at low density [96-98]. We found that untreated CF spontaneously undergo this differentiation under normal culture conditions (see Materials and Methods), as shown by enhanced α-SMA expression in CF between passages 2 and 5 (Figure 3-2A). Thus adult rat CF convert to myofibroblasts during early passage when plated on plastic tissue culture dishes in serum-containing media, emphasizing the importance culture conditions when conducting studies of the effects of exogenous agents on myofibroblast formation. For this reason all differentiation studies were conducted under serum-free conditions using low passage CF (passage ≤ 2) that were plated at relatively high density (~200 cells/mm^2) .

We hypothesized that increases in cellular cAMP would inhibit myofibroblast differentiation. Cellular cAMP levels are determined in part by the expression and activity of phosphodiesterases (PDE's), which catalyze the hydrolysis of cAMP to AMP. To determine whether endogenous levels of cAMP are sufficient to prevent myofibroblast transformation, we examined α-SMA expression by CF maintained under normal culture conditions for up to 72 hr in the absence or presence of isobutylmethylxanthine (IBMX; 200 μM), a non-specific PDE inhibitor. We found that α-SMA expression increases in a time-dependent manner under normal culture conditions and that this increase is blunted by addition of IBMX (Figure 3-2B).

To determine the impact of AC activation on myofibroblast formation, we investigated the effects of forskolin on basal (0 %FBS), TGFβ- or Ang II-stimulated α-SMA expression (Figure 3-2 C and D). Immunohistochemical analysis revealed

enhanced α-SMA staining and formation of prominent stress fibers by CF treated for 24 hrs with TGFβ (10 ng/ml) or Ang II (100 nM). Forskolin (10 μM) reduced basal, TGFβ- or Ang II- stimulated α-SMA expression, as assessed by immunohistochemistry (Figure 3-2C) or by immunoblot analysis (Figure 3-2D). Treatment of CF with TGFβ or Ang II significantly ($p<0.05$) enhanced α-SMA expression (114 ± 18% and 58 ± 24%, respectively), increases that were reduced by addition of forskolin. Thus treatment with forskolin for 24 hr inhibits α-SMA protein expression in response to pro-fibrotic agents that induce myofibroblast transformation. Forskolin also produced a nearly 70% reduction in basal α-SMA protein expression, indicating that increases in cAMP can inhibit "spontaneous" myofibroblast transformation of CF.

AC6 overexpression enhances forskolin- and adrenomedullin-stimulated cAMP production and inhibition of serum-stimulated collagen synthesis by forskolin and adrenomedullin

We used an adenoviral vector to enhance expression in CF of AC6, an AC isoform expressed natively at relatively high levels [67]. Basal cAMP levels did not differ between LacZ- and AC6-overexpressing CF (Inset, Figure 3-3A), but cAMP production was substantially increased in response to both forskolin and adrenomedullin (Figure 3-3 A and B). Compared to LacZ controls, CF that overexpressed AC6 had enhanced cAMP production in response to forskolin with

more than 5-fold enhancement at 3 and 10 μM; maximal response to adrenomedullin was enhanced but its EC_{50} was similar in LacZ- and AC6-overexpressing CF (Figures 3-3 B and C). Thus, AC6-overexpression does not change basal cAMP levels in CF but enhances maximal cAMP formation by agents that stimulate AC, with no differences in cell viability at any agonist concentration.

AC6 overexpression in the absence of AC stimulants did not alter serum-stimulated collagen synthesis (Inset, Figure 3-4A) but enhanced forskolin- and adrenomedullin-mediated inhibition of collagen synthesis (Figure 3-4 and, B). This enhanced inhibition resulted from a lowering (> 10 fold) of the EC_{50} concentration for forskolin and an "uncovering" of inhibition of collagen synthesis by adrenomedullin at concentrations ≥ 10 nM (cf. the latter results with those in Figure 3-1A and 3-4B). Overexpression of AC6 thus substantially sensitizes adult rat CF to inhibition of collagen synthesis by both forskolin and adrenomedullin.

TGFβ treatment is known to enhance the pro-fibrotic state by altering expression of proteins such as plasminogen activator inhibitor (PAI-1) and interleukin-6 (IL-6) [25, 99]. Consistent with the ability of increased AC expression to blunt collagen synthesis, overexpression of AC6 decreased basal and TGFβ-regulated expression of PAI-1 by 89 ± 3% and 59 ± 8%, respectively and IL-6 by 81 ± 2% and 92 ± 3%, respectively.

Overexpression of AC6 enhances forskolin- or adrenomedullin-mediated inhibition of myofibroblast formation

To determine whether AC overexpression alters CF-to-myofibroblast transformation, we examined the effects of forskolin and adrenomedullin on TGFβ-stimulated α-SMA expression. Sub-maximal concentrations of forskolin and adrenomedullin were used because, as shown in Figures 3-4B and C, these concentrations produced the most profound differences in collagen synthesis between LacZ- and AC6-overexpressing CF. LacZ-overexpressing CF showed moderate α-SMA expression that was enhanced by treatment with TGFβ (10ng/ml); this enhancement was inhibited by treatment with forskolin (1 μM) but not adrenomedullin (0.1 μM) (Figure 3-5A and C). AC6 overexpression inhibited basal α-SMA expression and enhanced the ability of both forskolin and adrenomedullin to blunt TGFβ-stimulated α-SMA expression (Figure 3-5B and C). Consistent with the effects observed on collagen synthesis (Figure 3-1A and 3-4B), overexpression of AC6 "uncovered" a prominent inhibition of α-SMA expression by adrenomedullin. Similar trends in the intensity of F-actin staining with phalloidin were observed in response to forskolin and adrenomedullin (Figure 3-5A and B), suggesting that actin stress fiber formation is blunted in parallel with the decrease in α-SMA expression. Expression of vimentin, a marker for both fibroblasts and myofibroblasts [100], did not change during myofibroblast transformation. Overall, these data show that AC6-overexpression blunts basal α-SMA expression and enhances the ability of both forskolin and a G_S-coupled receptor agonist (adrenomedullin) to inhibit TGFβ-

stimulated α-SMA expression, thus attenuating the fibroblast-to-myofibroblast transformation in parallel with a decrease in collagen synthesis.

Discussion

Gabbianni et al. [101] first identified a modified form of granulation tissue fibroblasts that exhibited smooth muscle-like properties, suggesting a role for these cells in wound contraction during tissue repair. These fibroblasts, termed myofibroblasts, contribute to wound repair and connective tissue remodeling, in particular to establishment of tension and enhanced synthesis of ECM [32]. Although a number of hormones and growth factors are known to stimulate formation of myofibroblasts from fibroblasts, much less is known regarding approaches to blunt this transformation and the increased tissue fibrosis that results. Glucocorticoids are one class of agents that sometimes, but not always, inhibits myofibroblast formation and proliferation [102, 103], perhaps via blockade of TGFβ production [104]. Other therapies to blunt fibrosis are under study but none has been shown to be efficacious and non-toxic [105].

This is the first study, of which we are aware, to demonstrate the ability of AC activation to inhibit myofibroblast transformation of adult rat CF in a manner that correlates with a reduction in collagen synthesis. Our findings also show the importance of levels of AC expression in determining the ability of a GPCR agonist or forskolin to regulate myofibroblast formation and collagen production. Taken together, these data suggest that blockade of myofibroblast formation, via increases in

cAMP, blunts fibrosis by inhibiting generation of the cellular phenotype responsible for exaggerated ECM production.

Previous studies show that AC is the component in the GPCR-G-protein-AC pathway that limits agonist-mediated increases in maximal cAMP production, including in cardiac myocytes [45-47]. AC6, one of 9 membrane isoforms of AC, is expressed in many tissues, including CF, and is subject to inhibition by many factors, including $G_{\beta\gamma}$, $G_{\alpha i}$, Ca^{++}, protein kinase C and nitric oxide [48, 49]. We hypothesized that AC6-overexpression might sensitize CF to GPCR agonists that stimulate AC (without altering basal cAMP levels [47]) and thereby enhance the effects of AC activation on myofibroblast formation and collagen production. Consistent with our hypothesis, we found that cAMP production in response to a GPCR-agonist (adrenomedullin) or direct activation of AC (forskolin) was enhanced by AC6-overexpression, and that this enhanced cAMP production correlated with increased inhibition of collagen synthesis and decreased formation of myofibroblasts. In this regard, the data concerning adrenomedullin are of particular interest.

Adrenomedullin is an endogenous vasodilator and natriuretic peptide that was originally discovered in human pheochromocytoma [106] and has since been shown to exhibit a wide range of cardioprotective effects. *In vitro* studies have shown that adrenomedullin inhibits neonatal rat cardiac fibroblast proliferation and collagen production in a cAMP-dependent manner and, in addition, Ang II receptor expression and function [56, 107]. Ang II is pro-fibrotic for cardiac fibroblasts from multiple species, including those isolated from explanted human hearts [15, 90]. Gene delivery

of adrenomedullin attenuates cardiac and renal fibrosis in deoxycorticosterone acetate (DOCA)-salt hypertensive rats [108] and adrenomedullin-knockout mice exhibit increased fibrosis and elevated expression of TGFβ in both heart and kidney [109]. Unlike previous studies [56] that used CF isolated from neonatal rats, in our studies with adult rats we did not observe an inhibition of collagen synthesis unless we overexpressed AC6 (cf. Figures 1A and 4B). Consistent with previous findings, our finding emphasize the role of increased cAMP in the anti-fibrotic effects of adrenomedullin but our data further show the enhancement of these effects by AC6 overexpression and that the anti-fibrotic effects of adrenomedullin likely result from cAMP-mediated regulation of myofibroblast formation. These findings thus define a potential means to attenuate cardiac fibrosis by strategies that increase expression of AC6 in CF and sensitize cells to both endogenous and exogenous agonists, such as adrenomedullin, that increase cAMP formation.

The precise mechanisms by which AC activation inhibits myofibroblast formation will require further study. A primary component of the fibroblast-to-myofibroblast transformation is the formation of a contractile apparatus containing actin stress fibers, which are a defining characteristic of myofibroblasts (Figure 2B, 5A, 5B; [9]). Activation of the low molecular weight G protein RhoA plays a key role in proper formation and assembly of actin cytoskeleton [110, 111]. cAMP, through the activation PKA, can inactivate RhoA and promote loss of stress fibers [112, 113]. The inhibition of fibroblast-to-myofibroblast transformation by AC activation may result from an inhibition of RhoA and the contractile machinery that defines a myofibroblast.

It is possible that other effects are involved, such as blockade of α-SMA production at the level of gene expression: a TGFβ response element in the α-SMA promoter is required for α-SMA gene expression [114, 115]. TGFβ signaling occurs via Smad proteins and a requirement for Smad proteins, specifically Smad3, in TGFβ regulation of α-SMA gene expression has been suggested during myofibroblast transformation of rat lung fibroblasts [116]. Recent data indicate that cAMP acts in a PKA-dependent manner to inhibit TGFβ/Smad signaling and gene activation by disruption of transcriptional cofactor binding [117].

The phenomenon of myofibroblast formation from fibroblasts and its pro-fibrogenic role in connective tissue production is well conserved regardless of the tissue of residence [9, 32]. In the heart, one confronts this problem in the setting of cell injury and associated cardiac fibrosis, especially following myocardial infarction [3, 4, 118]. A major dilemma is how to limit continued ECM production in regions remote from the site of injury that can eventually lead to diminished contractile function. Agents that inhibit the fibroblast-to-myofibroblast transformation may provide a novel therapeutic means to inhibit maladaptive remodeling in the heart and other organs. The data shown here demonstrate that pharmacological activation of AC, in particular after overexpression of AC6, inhibits collagen synthesis by adult rat CF, in parallel with an inhibition of myofibroblast formation. The results provide a rationale for strategies to increase cAMP levels in cardiac fibroblasts, perhaps by pharmacological agents or via gene transfer techniques, the latter as a means to enhance the efficacy of circulating or

local factors that stimulate production of cAMP and thereby attenuate and prevent fibrosis in the heart.

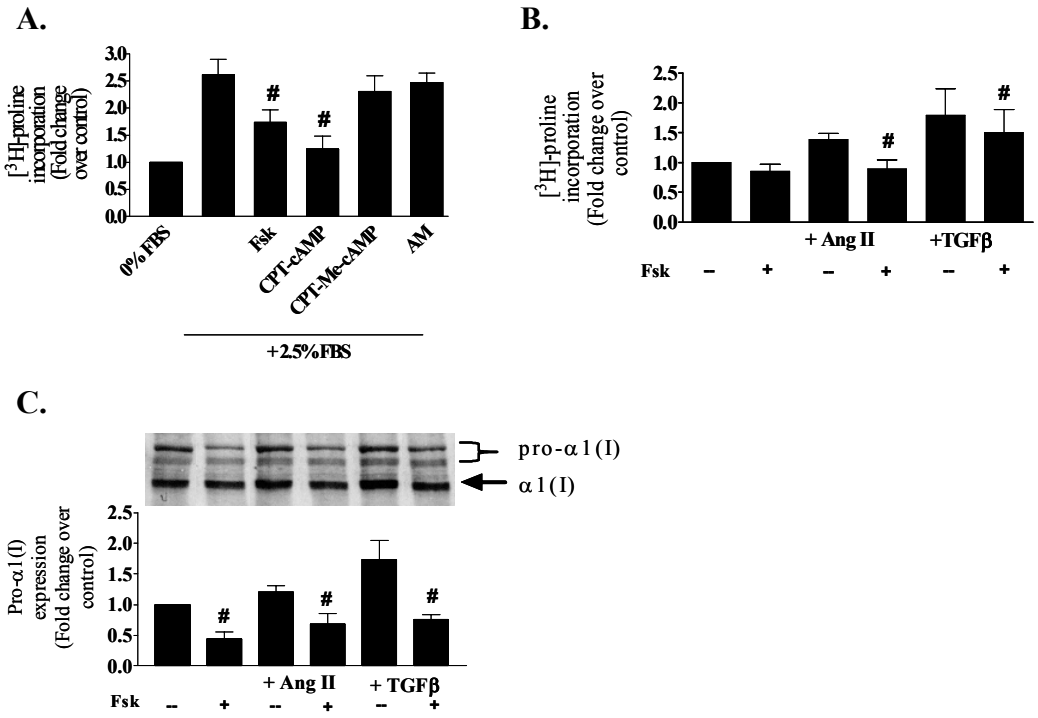


Figure 3-1. Forskolin and CPT-cAMP blunt collagen synthesis of adult rat cardiac fibroblasts. Collagenase-sensitive [^{3}H]-proline incorporation by fibroblasts grown for 48 hr in serum-free media and then stimulated for 48 hr with (A) serum-free media (control) or 2.5% in the absence or presence of forskolin (Fsk, 10 μM), CPT-cAMP (100 μM), CPT-Me-cAMP (100 μM) or adrenomedullin (AM, 1 μM) or (B) serum-free media alone or with Ang II (100 nM) or TGFβ (10 ng/ml) in the presence or absence of Fsk (10 μM). (C) Cardiac fibroblasts were cultured and serum-starved as noted above and subsequently treated with the indicated agents for 48 hr prior to protein isolation (see Materials and Methods for lysis buffer composition). Densitometry and quantitation for panel C was performed on the pro-α1 (I) band (top band). Values represent mean ± SEM of at least 4 experiments performed in triplicate and compared using a paired t-test. # denotes $p < 0.05$ in response to forskolin or CPT-cAMP

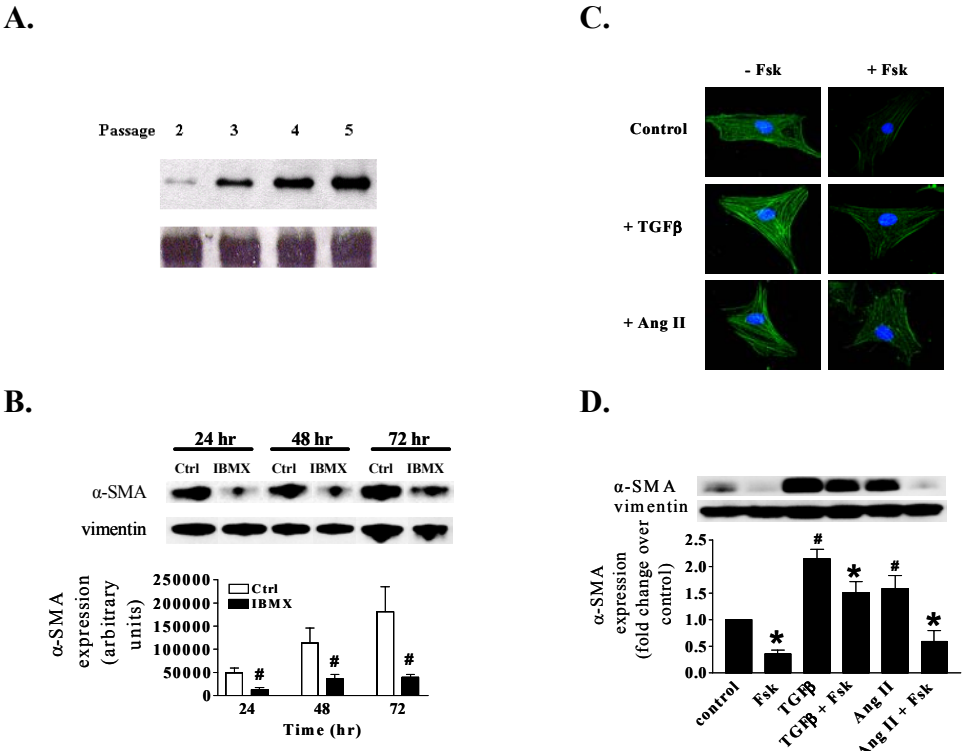
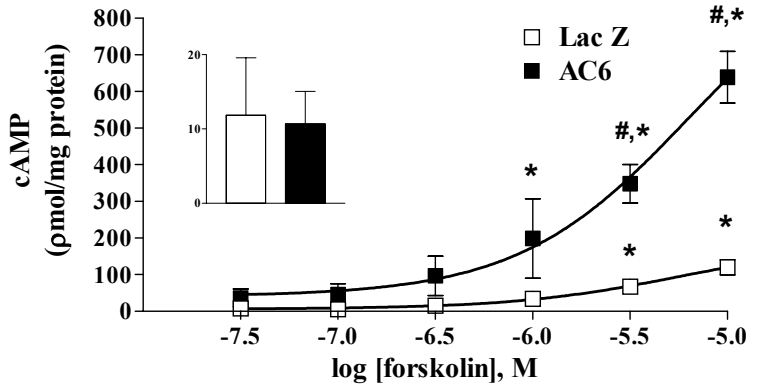


Figure 3-2. α -Smooth muscle actin (α-SMA) expression in CF of different passage number and in response to TGFβ and Ang II (A) α-SMA expression was measured by immunoblot analysis using adult rat CF at passages 2 through 5. CF at passage 1 were split from confluent monolayers at a ratio of 1:3 and maintained in the presence of 10% FBS for 48 hr prior to collection of whole cell lysates. After imunoblotting for α-SMA (upper panel), the blot was stripped and re-probed with GAPDH as a loading control (lower panel). (B) α-SMA expression was measured using CF maintained in the presence of 10% FBS, with and without isobutylmethylxanthine (IBMX; 200 μM). After imunoblotting for α-SMA, the blot was stripped and re-probed with vimentin as a loading control and quantitation of α-SMA immunoreactive bands was performed. (C) Immunohistochemistry was performed using CF (passage 2) grown for 48 hr in serum-free media and then for 24 hr in serum free media alone (control), Ang II (100 nM) or TGFβ (10 ng/ml) in the presence or absence of Fsk (10 μM). Cells were stained for α-SMA (green) and nuclear staining of DNA with DAPI (blue). (D) α-SMA protein expression with these same treatments was verified by immunoblot. Data are expressed as fold change relative to control. Values represent mean ± SEM of at least 3 experiments and compared using a paired t-test and one-way analysis of variance with post-hoc multiple comparison tests. # denotes $p<0.05$ as compared to control, * denotes $p<0.05$ in response to forskolin.

A.



B.

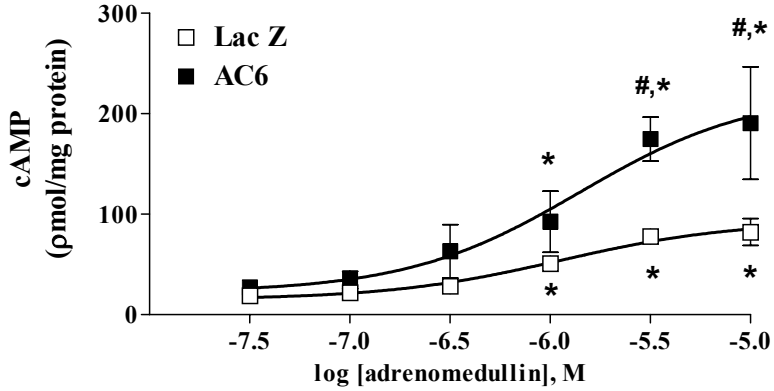


Figure 3-3.Overexpression of AC6 enhances forskolin- or adrenomedullin-stimulated cAMP production cAMP production was measured by radioimmunoassay using CF grown for 48 hr in serum-free media and then stimulated for 10 min with 2.5% fetal bovine serum (FBS) alone (A, inset) or in the presence of the indicated concentrations of (A, main panel) forskolin or (B) adrenomedullin. CF were incubated with an adenovirus expressing either LacZ (control) or AC6 for 24 hr prior to stimulation. Values represent mean ± SEM of at least 3 experiments and compared using a paired t-test and one-way analysis of variance. * denotes $p<0.05$ compared to 2.5% FBS, # denotes $p<0.05$ compared to LacZ cells.

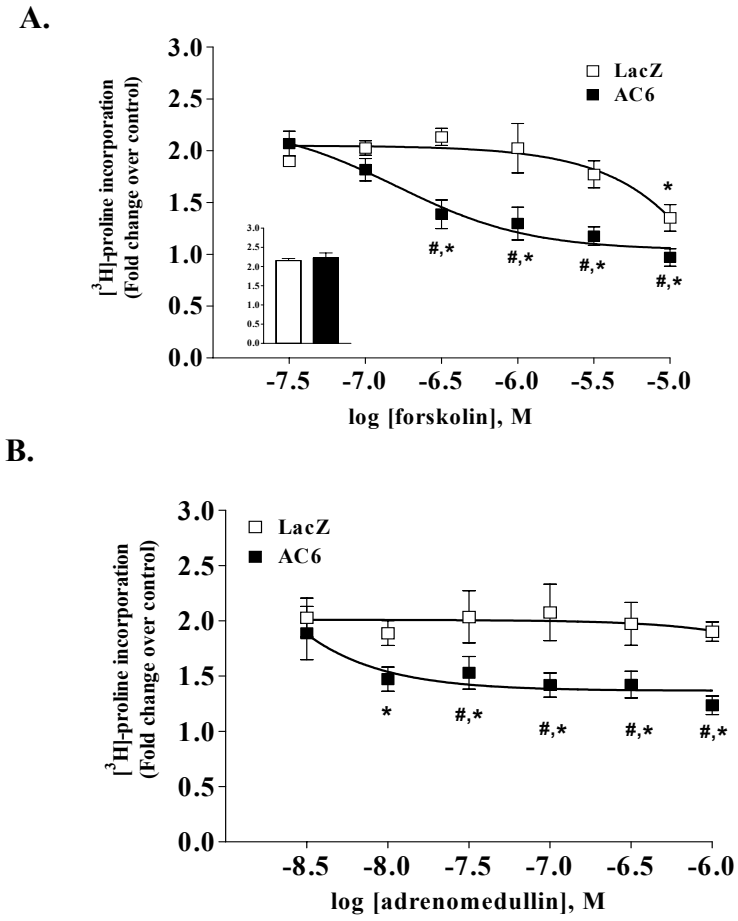


Figure 3-4. Overexpression of AC6 enhances forskolin- and adrenomedullin-promoted inhibition of collagen synthesisCollagenase-sensitive [^{3}H]-proline incorporation was measured using adult rat CF grown for 48 hr in serum-free media and then stimulated for 48 hr with 2.5% fetal bovine serum (FBS) in the absence (A, inset) or presence of the indicated concentrations of forskolin (A, main panel) or adrenomedullin (B). CF were incubated with an adenovirus expressing either LacZ (control) or AC6 for 24 hr prior to stimulation. Data are normalized for [^{3}H]-proline incorporation into cells grown under serum-free conditions. Values represent mean ± SEM of at least 3 experiments and compared using a paired t-test and one-way analysis of variance. * denotes $p<0.05$ compared to 2.5% FBS, # denotes $p<0.05$ compared to LacZ cells.

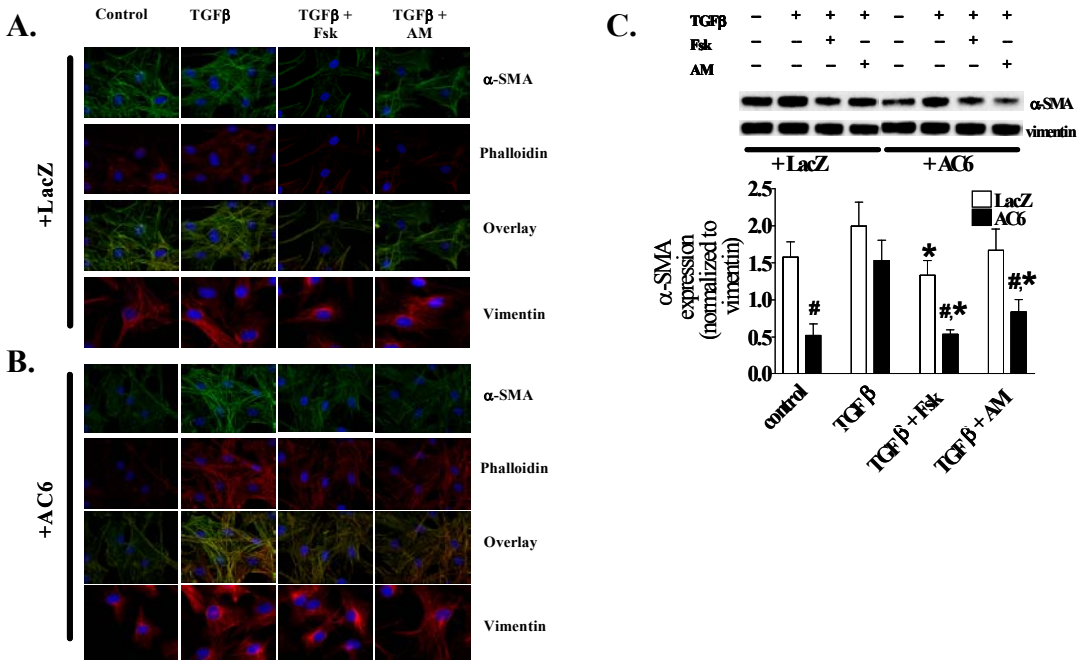


Figure 3-5. AC6 overexpression enhances forskolin- and adrenomedullin-mediated inhibition of TGFβ-stimulated α-SMA expression. Immunohistochemistry was performed using adult rat CF that overexpressed either LacZ (A) or AC6 (B). CF (passage 2) were grown for 48 hr in serum-free media and then for 24 hr in serum free media alone (control) or with TGFβ (10 ng/ml) in the presence or absence of forskolin (Fsk, 1 μM) or adrenomedullin (AM, 0.1 μM). CF were incubated with an adenovirus expressing either LacZ (control) or AC6 for 24 hr prior to stimulation. For immunohistochemical studies CF were stained for α-SMA (green), F-actin (phalloidin, red) Vimentin (red) and DNA (DAPI, blue). Overlap between α-SMA and F-actin staining is also represented (yellow). (C) α-SMA and vimentin protein expression in these same treatments were verified by immunoblot analysis. Quantitation of immunoreactive bands was performed; α-SMA expression was normalized to that of vimentin. Values represent mean ± SEM of at least 4 experiments and compared using a paired t-test. * denotes $p<0.05$ compared to TGFβ, # denotes $p<0.05$ compared to LacZ cells.

This chapter, in full, is a reprint or has been submitted for publication of the material as it appears in the Proceedings of the National Academy of Sciences, 2005 Jan;102(2):427-442., Swaney, James. S.; Roth, David M.; Olson, Eric R.; Naugle, Jennifer E.; Meszaros, J.Gary;, Insel, Paul A. The dissertation author was the primary investigator and single author of this paper.

Chapter 4

Scaffolding of adenylyl cyclase with phosphorylated caveolin-1 in focal adhesion complexes regulates fibroblast-myofibroblast transformation

Abstract

Fibroblast-myofibroblast transformation, a critical event for enhanced extracellular matrix deposition, involves formation of an actin stress fiber contractile apparatus that anchors and radiates from focal adhesions (FA) in the plasma membrane. Activation of adenylyl cyclase (AC, i.e. increases in cAMP) negatively regulates such transformation. Caveolae and their unique protein caveolins serve as scaffolds for signaling molecules, including AC isoforms, while phosphorylated caveolin-1 (phospho-cav-1) may localize at FA. Here, we used adult rat cardiac fibroblasts to examine distribution and expression of AC, phospho-cav-1 and FA proteins in order to define mechanisms that link increases in cAMP to caveolin-1 phosphorylation, actin/FA assembly and fibroblast-myofibroblast transformation. Sucrose density gradient centrifugation, immunoblot and immunohistochemical analysis revealed that, unlike cav-1, phospho-cav-1 is enriched in membrane fractions that also express FA proteins localized at the ends of actin stress fibers. AC expression was detected in both cav-1 and phospho-cav-1 immunoprecipitates, whereas FA kinase (FAK), phospho-FAK (FAK Y397), paxillin and vinculin were only in phospho-cav-1 immunoprecipitates. Treatment with the AC activator forskolin or a cAMP analog (CPT-cAMP) increased cav-1 phosphorylation but decreased FAK Y397 phosphorylation in a PKA-dependent manner, effects that preceded disruption of the actin cytoskeleton. Inhibition of protein tyrosine phosphatase 1B abrogated cAMP-mediated disruption of actin cytoskeleton, cav-1 phosphorylation and FAK Y397 dephosphorylation. The data thus define a novel organization of signaling molecules

that regulate fibroblasts: scaffolding of AC by phospho-cav-1 at FA sites in a caveolae-free microdomain that also possesses components that mediate inhibition of actin/FA assembly and fibroblast-myofibroblast transformation via increases in cAMP.

Introduction

Fibroblast-myofibroblast transformation is a key event in the deleterious remodeling that results in exaggerated production of connective tissue following injury of the lung, liver, kidneys, skin and heart [1, 34-36]. Relatively little is known regarding the specific cellular mechanisms that lead to myofibroblast formation, although a primary component is the formation of a contractile apparatus composed of α-smooth muscle actin (SMA)-containing microfilaments; ie., stress fibers, that anchor and radiate from FA along the plasma membrane [9]. Recent evidence suggests that FA serve as organizing centers for regulatory and structural proteins so as to facilitate rapid, precise control of cell proliferation, differentiation and function [42, 44]. Among these are anchoring proteins, such as vinculin, paxillin, talin and α-actinin, which link the actin cytoskeleton to transmembrane integrin receptors at FA [40]. Formation of focal contacts involves tyrosine phosphorylation of the non-receptor protein tyrosine kinase, FAK [41]. In response to growth factor stimulation and integrin engagement, FAK is autophosphorylated on Tyr397 [42], providing a docking site for Src kinase, which phosphorylates additional tyrosine residues in the FAK catalytic domain, resulting in full activation of FAK [43]. Phosphorylation at

Tyr397 also couples FAK to downstream signaling pathways that regulate cell proliferation, survival and motility as well as fibroblast-myofibroblast transformation [44, 63, 64].

Caveolae are cholesterol and sphingolipid-rich, flask-like invaginations of the plasma membrane that serve as organizing centers for certain transporters, receptors and post-receptor signaling components, facilitating rapid, coordinated regulation of cell function [72-74]. Caveolae contain unique proteins (e.g., caveolin-1, -2, and –3), with specific regions in their primary sequence that scaffold and organize signaling molecules [119]. In addition to their role as scaffolding proteins, caveolins-1 and -2 (cav-1 and cav-2) are substrates for phosphorylation by tyrosine kinases such as Src family kinase [75, 76, 120], in the case of cav-1 on tyrosine 14. [75, 78, 79]. While unphosphorylated cav-1 randomly distributes within the cell membrane [74], phosphorylated caveolin-1 (phospho-cav-1) localizes primarily in close proximity to FA. [77, 78]. The functional activity of phospho-cav-1 is poorly understood but its localization near FA and associated microfilaments suggests a role in the regulation of actin and FA dynamics and potentially in the modulation of fibroblast-myofibroblast transformation.

Adenylyl cyclases (AC) are membrane-bound proteins that catalyze conversion of ATP to cyclic AMP (cAMP), a ubiquitous second messenger that has numerous effects on cell function and morphology, primarily (albeit not exclusively [95]) through the activation of cAMP-dependent protein kinase, PKA. One such effect is to blunt FAK phosphorylation and to induce disassembly of actin stress fibers and FA

[65]. Other studies implicate cAMP-promoted PKA activation in the phosphorylation of cav-1 as a prelude to cell rounding [121]. Our recent studies provided evidence that AC activation or cAMP analogs that activate PKA inhibit cardiac myofibroblast formation via effects on α-SMA formation [82]. To date, though, no evidence has linked AC and phospho-cav-1 in terms of effects on cell morphology or function.

Here, we set out to test such a linkage. We find that in adult rat cardiac fibroblasts (CF) phospho-cav-1 colocalizes with AC at FA at plasma membrane sites that are independent of caveolae or lipid rafts. We show that stimulation of AC or incubation with a cAMP analog increases phosphorylation of cav-1 but decreases FAK Y397 phosphorylation in parallel with a disruption of basal and TGFβ-stimulated actin and FA assembly. We further show that protein tyrosine phosphatase (PTP) inhibition abolishes the effects of increased cAMP on cell morphology. These data thus provide unique evidence that phospho-cav-1-mediated scaffolding of AC at FA sites, independent of caveolae, facilitates rapid dysregulation of actin and FA assembly, thereby leading to inhibition of fibroblast-myofibroblast transformation.

Materials and Methods

Antibodies and Reagents

α-SMA, vimentin, paxillin and phospho-FAK (Tyr397) were purchased from Sigma. Total FAK, phospho-cav-1 (Tyr14) and cav-1 (mouse monoclonal and rabbit polyclonal) antibodies were purchased from Transduction Labs. AC5/6 antibodies were purchased form Santa Cruz. Alexa Fluor 647 Phalloidin probe for F-actin was

obtained from Molecular Probes. The src kinase inhibitor (PP2; 4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine), cAMP analog (8-CPT-cAMP; Adenosine 3',5'-cyclic monophosphate, 8-(4-Chlorophenylthio)-Sodium Salt), PKA inhibitor (Rp-cAMPS; Adenosine 3',5'-cyclic Phosphorothiolate-Rp) and PTP1B inhibitor (3-(3,5-Dibromo-4-hydroxy-benzoyl)-2-ethyl-benzofuran-6-sulfonicacid-(4-(thiazol-2-ylsulfamyl)-phenyl)-amide) were purchased from Calbiochem. All other drugs and reagents were obtained from Sigma.

Isolation and Culture of Adult Rat CF

CF were isolated from adult Sprague-Dawley rats (250-300 g, male) and cultured as previously described [82].

Membrane Fractionation

CF were fractionated using a modification of a detergent-free method [70, 122]. CF from two 10 cm plates were washed twice in ice-cold PBS and scraped in 1 ml of 150 mM sodium carbonate (pH 11.0) containing 1 mM EDTA, protease inhibitor cocktail (Sigma) and phosphatase inhibitor cocktail (Calbiochem). Cell lysates were sonicated on ice with three cycles of 20 sec bursts. Approximately 1 ml of homogenate was mixed with 1 ml of 80% sucrose in 25 mM MES, 150 mM NaCl (MBS, pH 6.5) to form 40% sucrose and loaded at the bottom of an ultracentrifuge tube. Discontinuous sucrose gradients (generated by layering 6 ml of 35% sucrose prepared in MBS followed by 4 ml of 5% sucrose) were centrifuged at 175,000 g

using a SW41Ti rotor (Beckman) for 3 h at 4°C. Samples were removed in 1 ml aliquots to form 12 fractions.

Immunoprecipitation, immunohistochemical and immunoblot analysis of CF

Immunoprecipitations were performed using either protein A or protein G-agarose (Roche). CF from a 10 cm plate were washed twice in ice-cold PBS and scraped in 1 ml of lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Igepal) containing protease inhibitor cocktail (Sigma) and phosphatase inhibitor cocktail (Calbiochem). Lysates were pre-cleared with protein-agarose for 1-3 h at 4°C, incubated with primary antibody for 1-3 h, immunoprecipitated with protein-agarose overnight at 4°C and centrifuged at 13,000 g for 5 min. Protein-agarose pellets were washed once in lysis buffer followed by subsequent washes in wash buffer 2 (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.2% Igepal CA-630) and wash buffer 3 (10 mM Tris-HCl, pH 7.5, 0.2% Igepal CA-630). Immunoblot and immunohistochemical (with image deconvolution) analyses were conducted as described [82].

Data analysis

Statistical comparisons and graphical representation were performed using Graph Pad Prism 3.0 (GraphPad Software). Statistical significance was set at $p<0.05$.

Results

Phospho-cav-1 localizes at FA sites, independent of caveolae, in adult rat CF

Using immunohstochemistry and immunoblot analysis, we examined the localization of cav-1 and phospho-cav-1 in adult rat CF. Although cav-1 was distributed throughout the plasma membrane, phospho-cav-1 localized primarily at FA sites that were independent of caveolae (Fig.4-1A), as identified by cav-1 staining. Phospho-cav-1 localized at the end of actin microfilaments in a manner identical to FAK (Fig. 4-1A, upper panels) and the FA markers paxillin and vinculin (data not shown), whereas cav-1 did not localize with either FAK or paxillin at FA sites (Fig. 4-1A, bottom middle and right panels). Phospho-cav-1 also exhibited a high degree of colocalization with tyrosine phosphorylated FAK (FAK Y397) (Fig. 4-1A, bottom left), which is generated by autophosphorylation at FAK following integrin engagement, making FAK Y397 a highly specific FA marker [41]. To confirm interaction between phospho-cav-1 and FA proteins, pull-down assays were conducted using cav-1 and phospho-cav-1 antibodies and immunoprecipitates were probed for expression of FAK, FAK Y397, paxillin and vinculin (Fig. 4-1B). Detection of cav-1 in immunoprecitates confirmed successful pull down. FA proteins were detected almost exclusively in phospho-cav-1 immunoprecipitates with little or no detection in cav-1 immunoprecipitates. These findings suggest that, unlike cav-1, phospho-cav-1 localizes predominantly at FA sites where it interacts with multiple FA proteins.

In order to determine the relative distribution of cav-1 and phospho-cav-1 in buoyant/lipid rafts vs.” heavy” membrane fractions, we conducted discontinuous

sucrose density gradient fractionation of CF cell lysates and quantitated cav-1 and phospho-cav-1 expression in buoyant (fractions 4-5) and heavy (fractions 10-12) membranes (Fig. 4-1C). Although cav-1 and phospho-cav-1 were present in both buoyant and heavy membrane fractions, expression of phospho-cav-1 was much greater in the heavy fractions, consistent with a non-caveolar distribution. FAK, FAK Y397, paxillin and vinculin were only detected in the heavy cellular fractions (Fig. 4-1D). These findings demonstrate that phospho-cav-1 is localized primarily at FA sites independent of caveolae/buoyant lipid rafts.

AC5/6 localizes with cav-1 throughout the cell membrane and with phospho-cav-1 at FA sites.

Previous work has shown that the majority AC5/6 is detected in buoyant/caveolar membrane fractions but a portion of AC5/6 localizes in heavy/non-caveolar membrane fractions in cardiac cells [67, 123]. Immunohistochemical analysis of CF revealed that, in addition to being distributed with cav-1 throughout the plasma membrane, a portion of AC 5/6 colocalized with phospho-cav-1 at FA sites (Fig. 4-2A). We verified this interaction by co-immunoprecipitation using AC 5/6 and phospho-cav-1 antibodies (Fig. 4-2B). AC5/6 enriches in the buoyant/caveolar fraction (the buoyant fraction contains only 5% of the protein found in the heavy fraction) but is also found in heavy fractions where phospho-cav-1 is located (Fig. 4-2C). Thus, a portion of AC5/6 colocalizes with and may be scaffolded by phospho-cav-1 at FA sites in adult rat CF.

AC activation or a cAMP analog stimulates cav-1 phosphorylation in a Src kinase- and PKA- dependent manner.

We examined whether increasing cAMP levels by AC activation using forskolin (Fsk, 10 μM) or by use of a cAMP analog (CPT-cAMP, 100 μM) affects cav-1 phosphorylation in CF. Addition of forskolin or CPT-cAMP induced time-dependent increases in cav-1 phosphorylation (Fig. 4-3A). Pretreatment of CF for 30 min with a Src kinase inhibitor (PP2; 10 μM) or a PKA inhibitor (Rp-cAMPs; 100 μM) abolished the forskolin-stimulated increase in phospho-cav-1 (Fig. 4-3B), demonstrating that cAMP acts in both a Src kinase- and PKA-dependent manner to increase phospho-cav-1. The stimulatory effects of AC/cAMP on phospho-cav-1 may result from scaffolding of AC 5/6 by phospho-cav-1 at FA sites, thereby providing a localized feedback mechanism for increased caveolin phosphorylation in response to AC activation.

cAMP/PKA-mediated cav-1 phosphorylation precedes disruption of actin cytoskeleton

Based on AC/cAMP-mediated inhibition of myofibroblast transformation [82], we hypothesized that scaffolding of AC 5/6 by phospho-cav-1 at FA sites may facilitate actin cytoskeleton disassembly. Using immunohisotchemistry, we examined the kinetics of cav-1 phosphorylation and actin reorganization following stimulation of CF with forskolin (10 μM) (Fig. 4-4). Within 5 min phospho-cav-1 intensity was dramatically increased and remained elevated for 30 min. By 30 min the actin

cytoskeleton began to breakdown, undergoing complete disruption after 60 min of forskolin stimulation. Similar to previous findings [124], the disruption of the actin cytoskeleton between 30-60 min coincided with a reduction in phospho-cav-1 intensity and a redistribution of phospho-cav-1 toward the interior of the cell.

cAMP/PKA-mediated disruption of actin cytoskeleton and FA assembly involves decreased FAK activation.

Increased FAK Y397 phosphorylation is implicated in fibroblast-myofibroblast transformation [63, 64]; thus, we investigated whether scaffolding of AC 5/6 at FA sites affects FAK phosphorylation in CF. Addition of forskolin (10 μM) or CPT-cAMP (100 μM) induced time-dependent decreases in FAK Y397 phosphorylation (Fig. 4-5A), which depended upon PKA, as indicated by blockade with Rp-cAMPs (100μM; Figure 4-5B). Forskolin treatment disrupted TGFβ (10 ng/ml)-stimulated assembly of actin stress fibers and FA complexes (Fig. 4-5C). The inhibitory effects of cAMP/PKA on fibroblast-myofibroblast transformation may thus be a consequence of dephosphorylation of FAK.

cAMP-mediated cytoskeleton disruption, cav-1 phosphorylation and FAK dephosphorylation are abrogated by a protein tyrosine phosphatase 1B (PTP1B) inhibitor.

Because both cav-1 and FAK are phosphorylated on tyrosine residues, we examined the role of protein tyrosine phosphatase (PTP) activity in cAMP-mediated

disruption of the actin cytoskeleton, increased cav-1 phosphorylation and FAK dephosphorylation by treating CF with forskolin in the presence of PTP inhibitors. Non-specific PTP inhibition with vanadate dose-dependently abolished the ability of forskolin to disrupt actin and focal adhesion assembly (Fig. 4-6A). Selective inhibition of PTP1B caused identical effects, suggesting a role for PTP1B in the cAMP-mediated effects on CF morphology. Inhibition of PTP1B reduced the ability of forskolin to stimulate cav-1 phosphorylation (Fig.4-6B) and dephosphorylate FAK Y397 (Fig. 4-6C). Together these results imply that cAMP-mediated disruption of the actin cytoskeleton and FA assembly involves PTP1B-mediated regulation of cav-1 and FAK Y397 phosphorylation.

Discussion

First identified in its phosphorylated form in v-src transformed cells [125], cav-1 has well-characterized effects on cell morphology and disease [72, 126] but little is known regarding the biological role of phospho-cav-1. Cav-1 is phosphorylated on tyrosine 14 in response to cellular stress, hormone and growth factor stimulation, and when phosphorylated, serves as a docking site for SH2 domain-containing proteins such as Grb7 [75]. The SH2 domain of Grb7 interacts with FAK through tyrosine 397 with subsequent effects on cell migration [80]. Based on its apparent localization near FA sites, phospho-cav-1 may be involved in stabilization of the actin cytoskeleton [127]. Phospho-cav-1 could therefore provide an anchoring site for proteins that control cell morphology and function via regulation of actin cytoskeleton dynamics.

We hypothesized that localization of phospho-cav-1 near FA sites, combined with the ability of cav-1 to scaffold AC, would provide a favorable organization of structural proteins and catalytic molecules to facilitate regulation of CF morphology and function by increased cAMP. Here, we demonstrate for the first time that phospho-cav-1 localizes at FA sites, independent of membrane caveolae, where it interacts with the FA proteins FAK, paxillin, and vinculin. At these sites, phospho-cav-1 scaffolds AC, which when activated, disrupts actin and FA assembly via desphosphorylation of the FA regulatory molecule FAK, with concomitant stimulation of cav-1 phosphorylation. Thus, cAMP-stimulated cav-1 phosphorylation appears to provide a positive feedback mechanism for increased scaffolding of AC (and perhaps other regulatory proteins) by phospho-cav-1 at FA sites during regulation of actin and FA dynamics (Figure 4-7).

AC/cAMP appears to possess the ability to dephosphorylate FAK and, in parallel, promote phosphorylation of cav-1 in CF. The fact that both FAK and cav-1 undergo PKA-dependent changes in tyrosine phosphorylation led us to hypothesize a regulation of protein tyrosine PTP activity by cAMP. Non-specific PTP inhibitors attenuate prostaglandin-mediated FAK dephosphorylation and actin disruption [65]. Phosphotyrosine phosphatase PTP1B exerts similar effects on actin cytoskeleton and FAK phosphorylation [128, 129] and can also promote the activity of src kinase via dephosphorylation of c-src tyrosine 529, the src autoinhibitory site [130]. Active src is able to promote of cav-1 phosphorylation [75, 127]. Since cAMP can stimulate PTP1B activity [131], we propose that PTP1B may be the downstream mediator for the

cAMP/PKA-mediated effects on phospho-cav-1, FAK and actin cytoskeleton in adult rat CF.

Consistent with this hypothesis, selective inhibition of PTP1B [132, 133] abolished the ability of cAMP/PKA to disrupt actin cytoskeleton and FA formation, phosphorylate caveolin-1 and dephosphorylate FAK (Fig 6). Overall, the current findings demonstrate a novel regulatory pathway whereby scaffolding and activation of AC at FA sites blocks FAK activation leading to rapid disruption of actin and FA assembly, events which depend upon PTP1B. Furthermore, cAMP/PKA-stimulated phosphorylation of cav-1 appears to potentiate these effects by positive feeding back on the increased scaffolding of AC by phospho-cav-1 at FA sites. These data thus suggest therapeutic strategies to inhibit fibroblast-myofibroblast transformation and deleterious organ fibrosis via cAMP/PKA-mediated regulation of actin and FA regulatory proteins at a unique membrane microdomain, the FA.

Currently, very few strategies exist for inhibiting deleterious ECM production associated with fibrotic disorders. The results here provide new mechanistic information regarding the ability of cAMP/PKA to control multiple downstream catalytic molecules in a manner that inhibits fibroblast-myofibroblast transformation, thereby suggesting potential targets to attenuate maladaptive connective tissue remodeling and organ fibrosis.

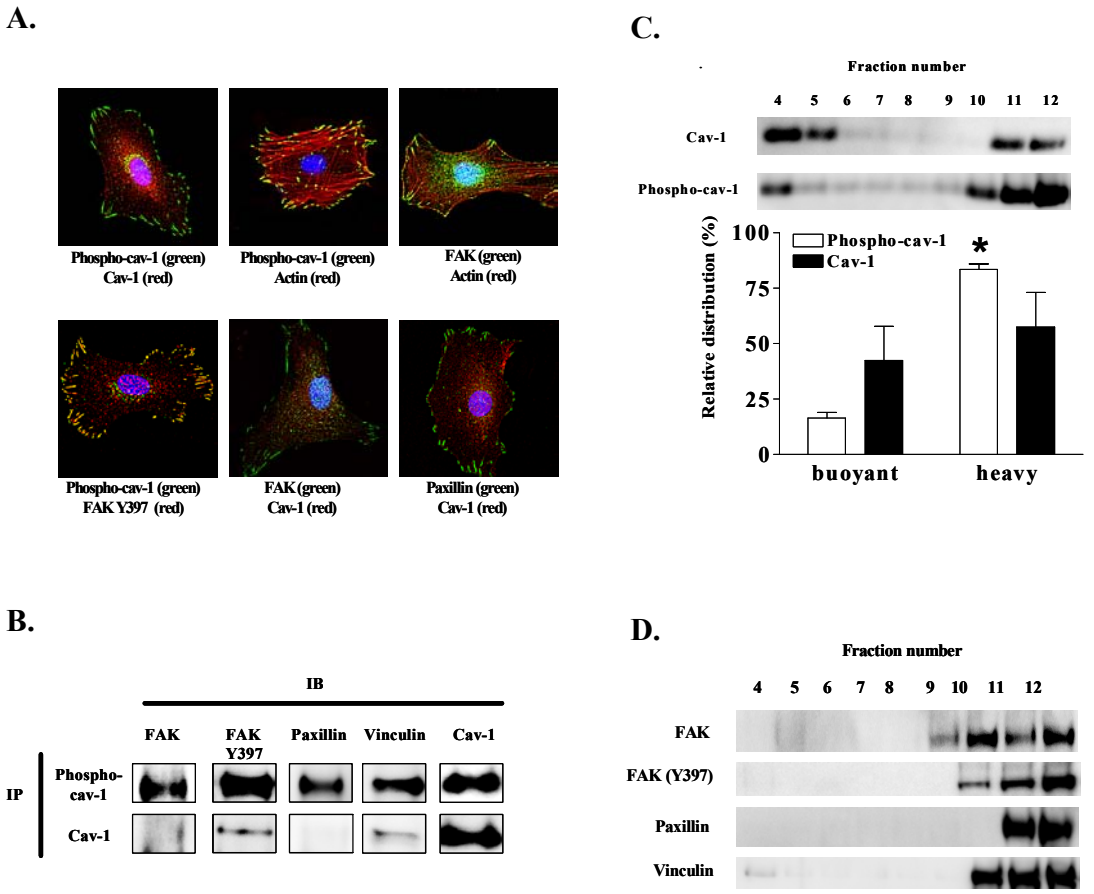


Figure 4-1. Phospho-caveolin-1 localizes primarily at focal adhesion sites, independent of caveolae, in CF. (A). Immunohistochemistry was performed using adult rat CF (passage <3) to localize cav-1, phospho-cav-1, FA kinase (FAK), FAK Y397, paxillin and F-actin (phalloidin) and nuclear staining of DNA with DAPI (blue). Colocalization is represented in yellow. (B) Colocalization was verified by immunoblot analysis of cav-1 and phospho-cav-1 immunoprecipitates to detect expression of FAK, FAK Y397, paxillin, vinculin and cav-1. (C) Relative distribution of cav-1 and phospho-cav-1 in buoyant/lipid raft (fractions 4-5) vs. heavy membrane (fractions 10-12) domains was determined using discontinuous sucrose gradient fractionation followed by immunoblot analysis and subsequent quantitation of immunoreactive bands. Values represent mean ± SEM of at least 4 experiments compared using two-way analysis of variance with post-hoc multiple comparison tests.* denotes $p<0.05$ between heavy and buoyant fractions. (D) Localization of FAK, FAK Y397 paxillin and vinculin was compared using discontinuous sucrose gradient fractionation followed by immunoblot analysis.

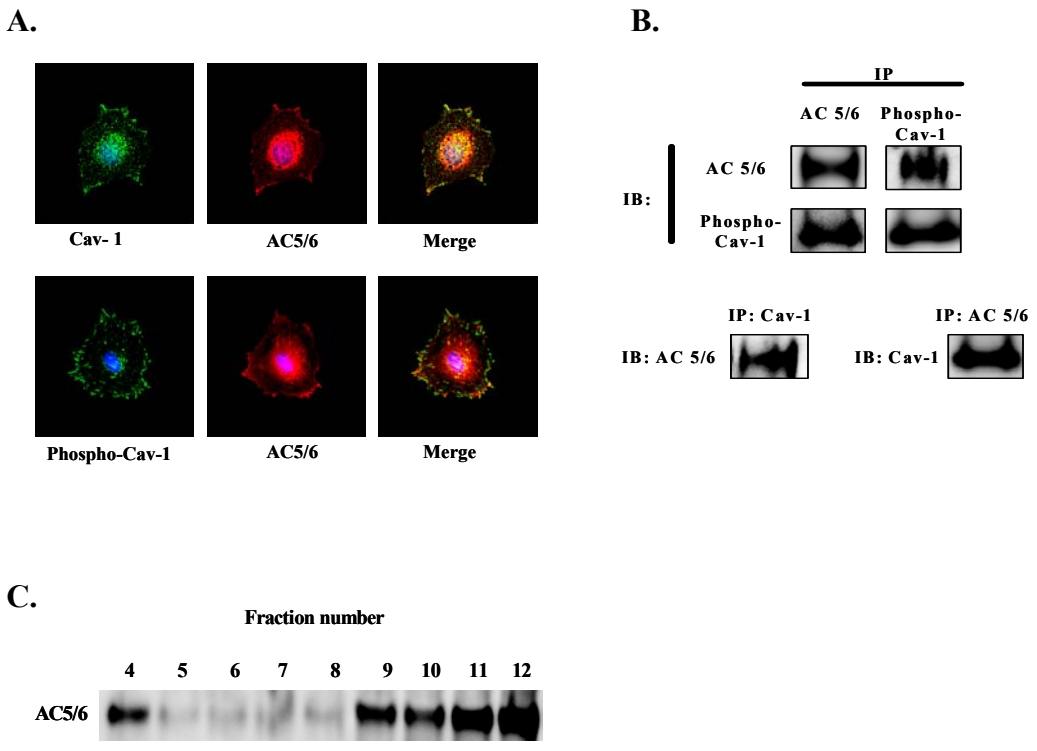


Figure 4-2. A portion of AC localizes with phospho-cav-1 at focal adhesion sites in CF. (A). Immunohistochemistry was performed using CF co-stained for cav-1 (green) or phospho-cav-1 (green) and AC5/6 (red) and nuclear staining of DNA with DAPI (blue). (B) AC 5/6 was detected by immunoblot (IB) in both cav-1 and phospho-cav-1 immunoprecipitates (IP). Cav-1 and phospho-cav-1 were detected in AC 5/6 immunoprecipitates. (C) Distribution of AC5/6 in both buoyant/lipid raft (fractions 4-5) and heavy membrane (fractions 10-12) domains was determined using discontinuous sucrose gradient fractionation followed by immunoblot analysis.

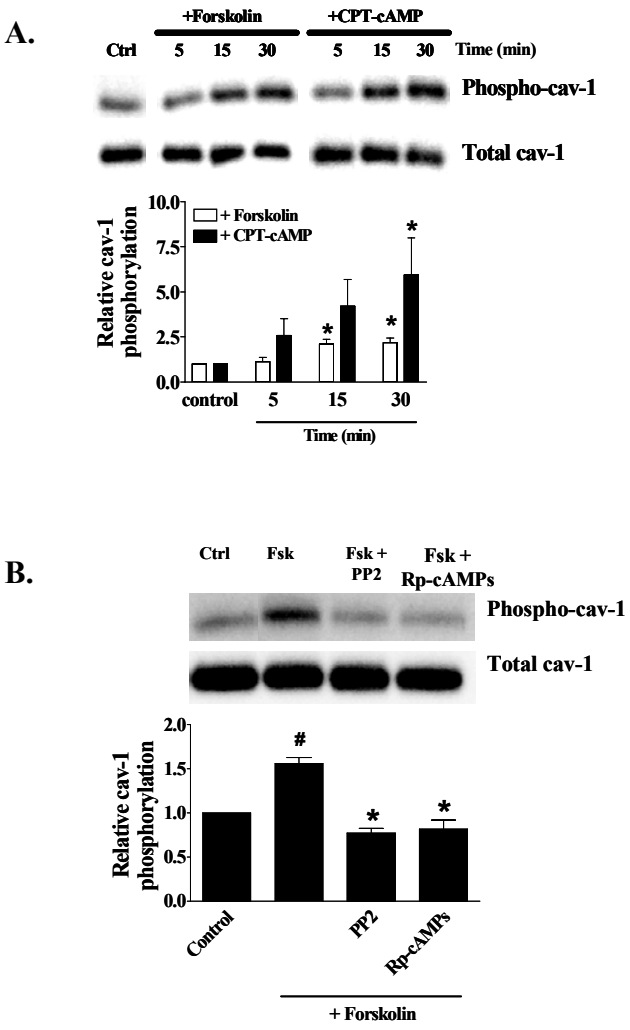


Figure 4-3.cAMP/PKA stimulates cav-1 phosphorylation in a Src kinase- and PKA-dependent manner. Adult rat CF grown for 48 hr in serum-free media and then stimulated with serum-free media alone (control) or for (A) 5, 15 and 30 min with forskolin (10 μM) or CPT-cAMP (100 μM) or (B) for 15 min with forskolin (10 μM) alone or in the presence of a Src kinase inhibitor (PP2; 10 μM) or a PKA inhibitor (Rp-cAMPs, 100 μM). Inhibitors were added 30 min prior to stimulation. Phospho-cav-1 and total cav-1 expression were determined by immunoblotting. Phospho-cav-1 levels were normalized for total cav-1 and data are expressed as fold change relative to control. Values represent mean ± SEM of at least 3 experiments and compared using a one-way analysis of variance with post-hoc multiple comparison tests. # denotes $p<0.05$ compared to control, * denotes $p<0.05$ compared to forskolin.

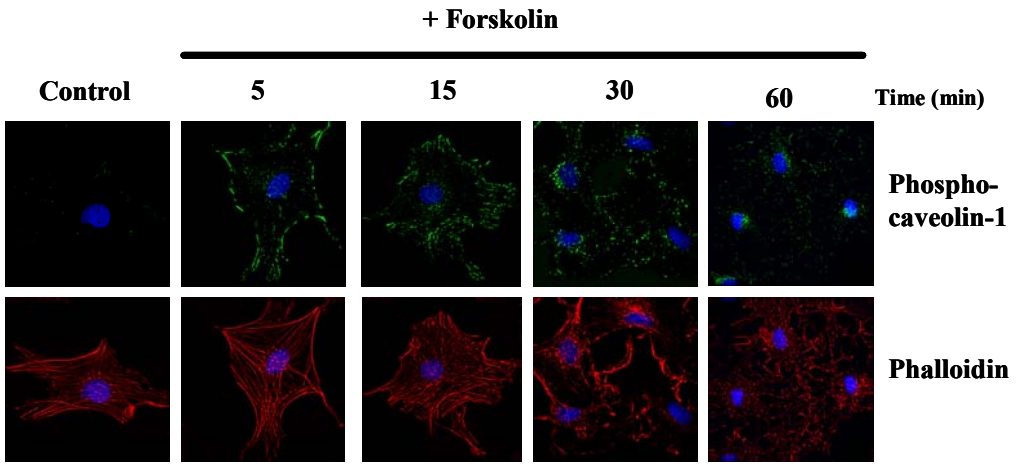


Figure 4-4.cAMP/PKA-stimulated cav-1 phosphorylation precedes disruption of the actin cytoskeleton. Immunohistochemistry was performed using adult rat CF grown for 48 hr in serum-free media and then stimulated with serum-free media alone (control) or for 5, 15, 30 and 60 min with forskolin (10 μM). CF were stained for phospho-cav-1 (green), F-actin (phalloidin, red) and DNA (DAPI, blue). Fluorescence intensity at all time-points was normalized relative to basal to account for increased cav-1 phosphorylation following stimulation.

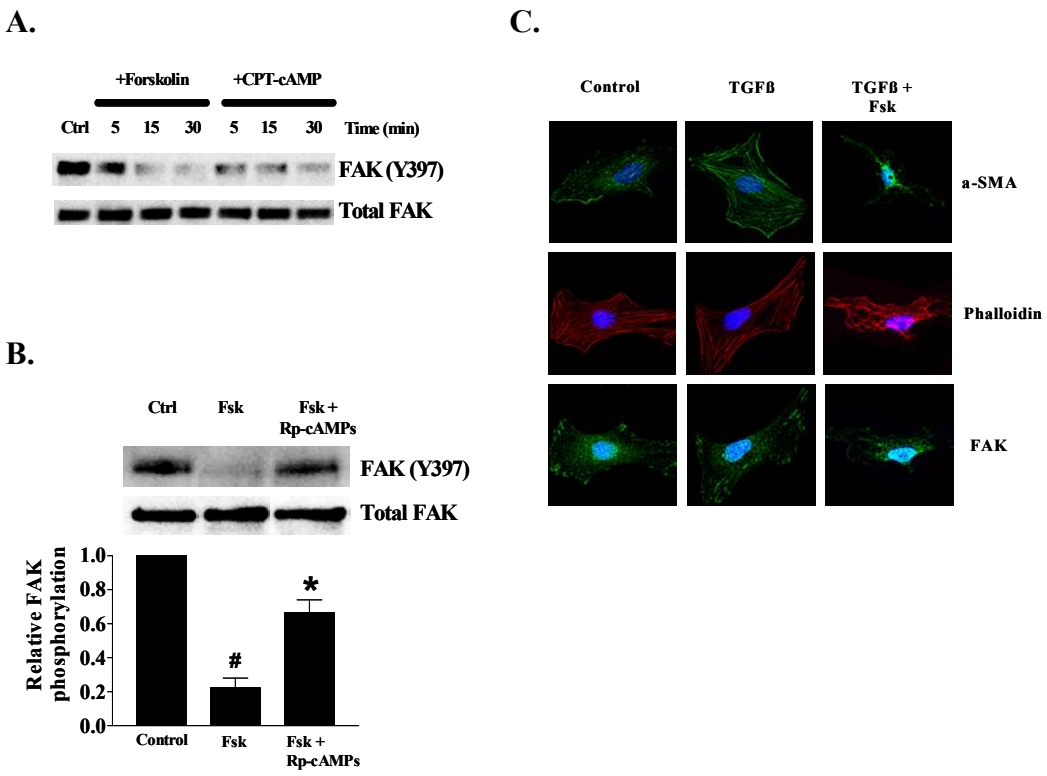


Figure 4-5.cAMP/PKA-mediated disruption of actin cytoskeleton and focal adhesion assembly involves decreased FAK activation. CF grown for 48 hr in serum-free media were stimulated with media alone (control) or for (A) 5, 15 and 30 min with forskolin (10 μM) or CPT-cAMP (100 μM) or (B) for 15 min with forskolin (10 μM) alone or in the presence of a PKA inhibitor (Rp-cAMPs, 100 μM). FAK (Y397) and total FAK were then determined by immunoblotting. Inhibitors were added 30 min prior to stimulation. Data were normalized for total FAK expression and expressed as fold change relative to control. Values represent mean ± SEM of at least 3 experiments and compared using a one-way analysis of variance with post-hoc multiple comparison tests. # denotes $p<0.05$ compared to control, * denotes $p<0.05$ compared to forskolin. (C) Immunohistochemistry was performed using CF grown for 48 hr in serum-free media and then stimulated for 60 min with media alone (control) TGFβ (10 ng/ml) alone or in the presence of forskolin (10 μM). CF were then co-stained for F-actin (phalloidin, red) and FAK (green) and nuclear staining of DNA (DAPI, blue).

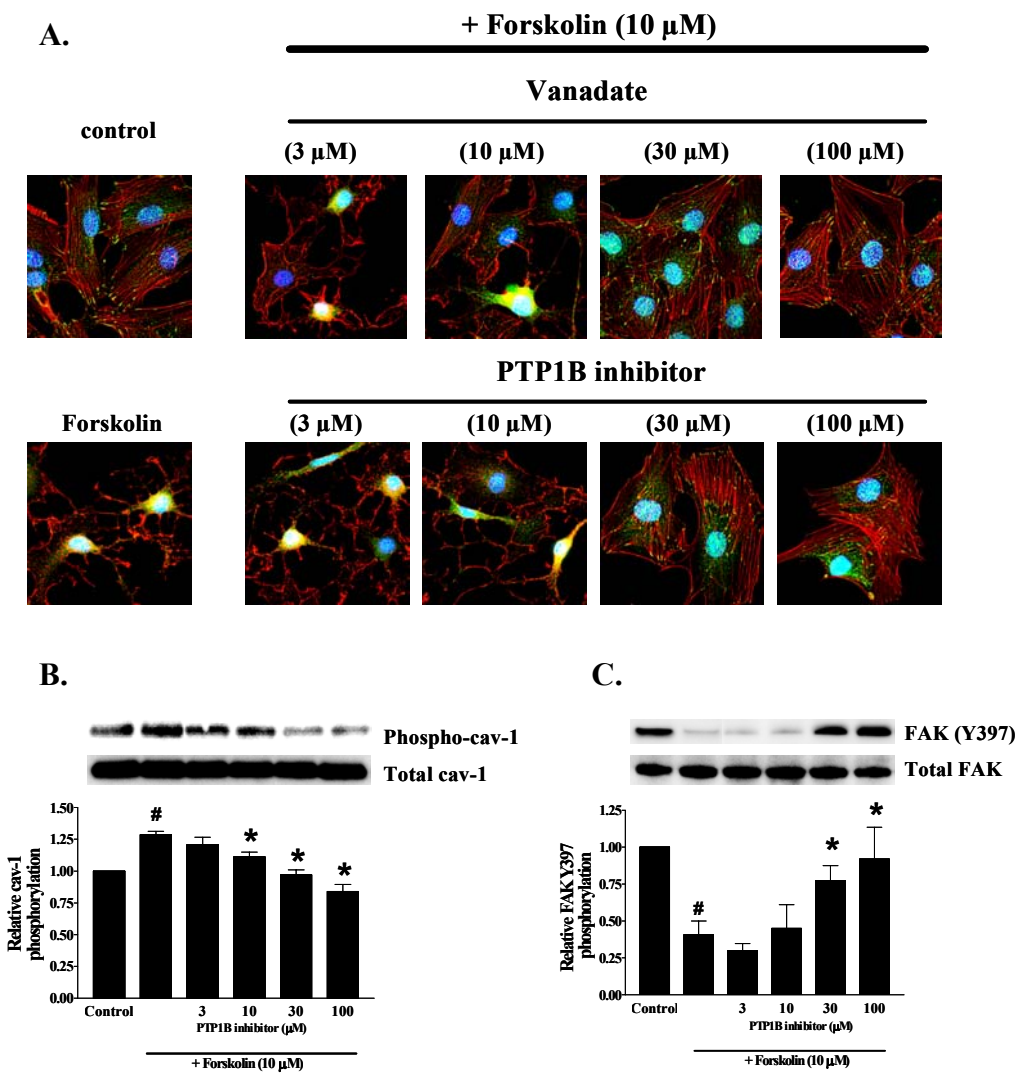


Figure 4-6.Protein tyrosine phosphatase (PTP) inhibition dose-dependently abrogates the effects forskolin on actin cytoskeleton, phospho-cav-1 and FAK Y397 (A). Immunohistochemistry was performed using CF grown for 48 hr in serum-free media and then stimulated for 60 min with serum-free media alone (control) or with forskolin (10 µM) in the absence or presence of the indicated concentrations of vanadate or PTP1B inhibitor. CF were then co-stained for FAK (green), F-actin (phalloidin, red) and DNA (DAPI, blue). The effects of PTP1B inhibition on (B) phospho-cav-1 and (C) FAK Y397 were examined using CF grown for 48 hr in serum-free media and then stimulated for 30 min with serum-free media alone (control) or with forskolin alone in the presence of a PTP1B inhibitor.

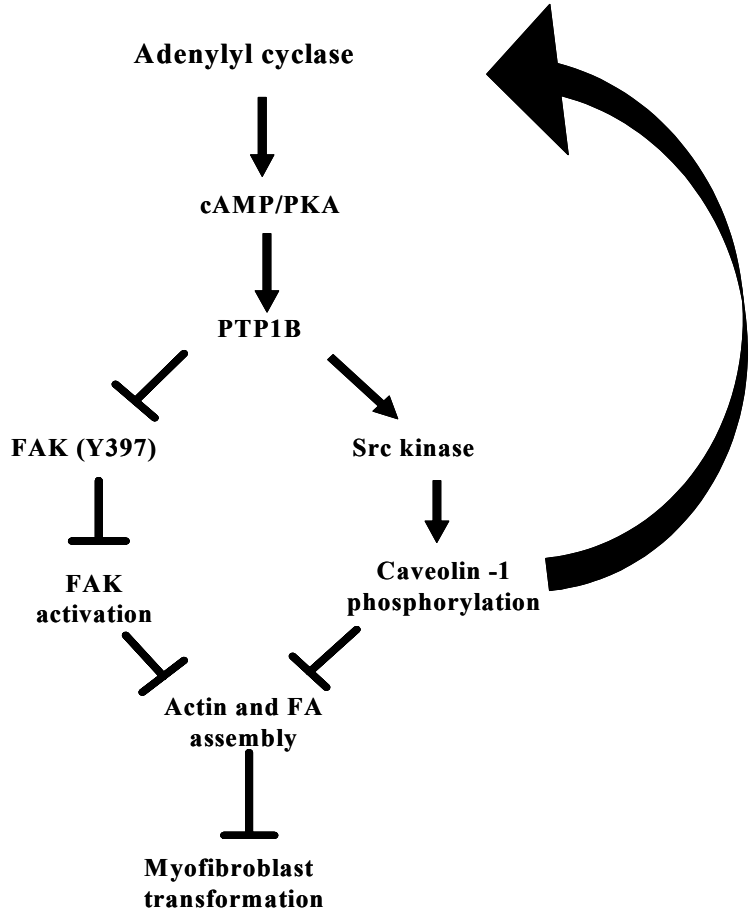


Figure 4-7. A proposed mechanism for PTP1B-dependent cav-1 phosphorylation and FAK Y397 dephosphorylation during cAMP/PKA-mediated inhibition of fibroblast-myofibroblast transformation. Stimulation of AC increases cAMP production and activates PKA, thereby activating PTP1B and resulting in: 1) dephosphorylation of FAK Y397 and inactivation of FAK and 2)activation of Src kinase [130] and increased phosphorylation of cav-1. These events result in disruption of actin and FA assembly and inhibition of fibroblast-myofibroblast transformation. cAMP/PKA-mediated phosphorylation of cav-1 provides a positive feedback mechanism that increases scaffolding of AC by phospho-cav-1 at FA sites and potentiates cAMP/PKA-mediated inhibition of fibroblast-myofibroblast transformation.

Chapter 5

Conclusion and Perspectives

Although it is well recognized that diastolic dysfunction is the underlying etiology in a large proportion of heart failure cases, very few strategies exist for attenuating the maladaptive connective tissue remodeling that contributes to myocardial stiffening and subsequent diastolic impairment. Current clinical strategies, involving receptor antagonists and biochemical inhibitors, function primarily at the extracellular level in an attempt to attenuate cardiac fibrosis during the late phase of remodeling after myocardial injury. However these treatments have achieved only limited success. Therefore, new information is needed to identify therapeutic strategies that inhibit cardiac fibrosis through regulation of post-receptor signaling and post-translational modification events in CF.

Activation and overexpression of adenylyl cyclase may represent a novel therapeutic approach to attenuate cardiac fibrosis through elevation of intracellular factors (ie., via cAMP) that regulate cellular function, proliferation and differentiation at the intracellular level. In the setting of heart failure following myocardial infarction, reparative fibrosis at the site of injury is critical to maintaining the structural integrity of the heart and avoiding cardiac rupture. However, diastolic dysfunction results from the late phase of scar formation whereby excess connective tissue is produced in the remaining, functional regions of the myocardium, reducing cardiac compliance and impairing the ability of the heart to relax and receive as well as eject blood. It is at this timepoint that AC may provide a beneficial therapeutic strategy to maintain cardiac function.

As I have shown in this thesis, AC expression and activity are decreased and collagen production is increased by CF late (18 weeks) after MI in rats. In light of the previous reports demonstrating the anti-fibrotic effects of AC/cAMP, downregulation of AC in the heart may contribute to diastolic dysfunction via a decreased capacity of CF to negatively regulate collagen production by increasing intracellular cAMP levels. In this setting, overexpression of AC6 in the heart through gene therapy may provide a beneficial approach for treatment of cardiac fibrosis. Increased AC expression sensitizes CF to AC agonists, thereby increasing maximal cAMP generation. By overexpressing AC in the heart after MI, circulating catecholamines and other cardioprotective factors that couple to G_S and activate AC may exhibit increased efficacy, in terms of cAMP generation, thereby attenuating fibroblast-myfibroblast transformation and collagen production by CF. A caveat to this approach is that, in order to be accepted as a treatment for cardiac fibrosis after MI, it would require a highly effective, non-invasive mode of delivery and sufficient level of cellular specificity. However these issues are currently being investigated.

Perhaps a more specific and potentially more effective means of blunting cardiac fibrosis might be to exploit the ability of AC/cAMP to inhibit fibroblast-myofibroblast transformation through regulation of FAK, PTP1B and even cav-1 phosphorylation. Each of these proteins represents a molecular target that is downstream of AC and is linked to AC-mediated regulation of cardiac myofibroblast formation. By taking a pharmacological approach to stimulate AC, or even regulate FAK, PTP1B and cav-1 directly, drugs might be developed that inhibit or possibly

even reverse formation of the pro-fibrotic myfibroblast phenotype. Thus, the current findings suggest targets that could be used as possible treatments for fibrotic disorders of the lung, liver, kidney and skin as well as the heart.

A limitation of the findings presented in this thesis is the limited data regarding the role of phosphodiesterases (PDE) in the cAMP/PKA-mediated effects on CF morphology and function. Intracellular cAMP levels are regulated by the activity of both the enzyme AC as well as cyclic nucleotide PDE's that catalyze its degradation. Herein, I have provided preliminary data demonstrating the ability of non-specific PDE inhibition (ie. IBMX) to attenuate fibroblast-myofibroblast transformation, presumably via decreased degradation of intracellular cAMP levels. However, comprehensive data regarding isoform-specific PDE expression and activity in CF would provide a more complete understanding of the capacity for cAMP generation and the ability to maintain intracellular cAMP levels through specific inhibition of PDE's expressed in CF.

In conclusion, I have taken a comprehensive approach to examine the role of AC in cardiac fibrosis at the whole animal level and to use subsequent *in vitro* methodologies to understand the cellular mechanism by which AC/cAMP regulates fibroblast-myofibroblast transformation. My hope is that these findings provide the foundation for an expanded body knowledge that may one day be used to develop effective clinical strategies for treating fibrosis in the heart as well as other organs.

Chapter 6

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