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Adenylyl Cyclase 2 Selectively Regulates IL-6 Expression in Human Bronchial Smooth Muscle Cells

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Abstract

Adenylyl cyclase (AC) catalyzes the formation of the ubiquitous second messenger cAMP. AC isoforms differ in their tissue distribution, cellular localization, regulation, and protein interactions, and most cells express multiple isoforms. We hypothesized that cAMP produced by different AC isoforms regulates unique cellular responses. Overexpression of individual isoforms had distinct effects on forskolin (Fsk)-induced expression of a number of known cAMP-responsive genes in human bronchial smooth muscle cells (BSMC) and human embryonic kidney cells (HEK-293). Most notable, in BSMC overexpression and activation of AC2 enhanced interleukin 6 (IL-6) expression, but overexpression of AC3 or AC6 had no effect. IL-6 production by BSMC was induced by Fsk and select G protein-coupled receptor (GPCR) agonists, though IL-6 levels did not directly correlate with intracellular cAMP levels. At low cAMP concentrations exchange protein directly activated by cAMP (Epac) predominated in mediating the IL-6 response, but at higher cAMP concentrations protein kinase A (PKA) assumed the larger role. IL-6 promoter mutations demonstrated that activator protein 1 (AP-1) and cAMP responsive element (CRE) transcription sites were required for cAMP mediated induction. Our findings indicate that AC2 participates in a cAMP-signaling compartment that specifically regulates IL-6 expression in BSMC and that other AC isoforms are excluded from this compartment.

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**Adenylyl Cyclase 2 Selectively Regulates IL-6 Expression in Human Bronchial
Smooth Muscle Cells**

A Dissertation
Presented for
The Graduate Studies Council
The University of Tennessee
Health Science Center

In Partial Fulfillment
Of the Requirements for the Degree
Doctor of Philosophy
From The University of Tennessee

By
Amy Sue Bogard
December 2013

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DEDICATION

Dedicated to the memory of Janie Van Prooijen who consistently offered tremendous support and encouragement to all IPBS students.

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I would like to thank my advisor Dr. Rennolds Ostrom for his thoughtful mentorship. He has made my time as a graduate student enjoyable and fulfilling, and on those days that I was completely overwhelmed, he always had just the right words to put me at ease. I would also like to thank my committee Dr. Elizabeth Fitzpatrick, Dr. Edwards Park, Dr. Shannon Matta, Dr. Steven Tavalin, and Dr. Christopher Waters for their guidance. Previous Ostrom lab members Joseph Kaminski and Dr. Muthusamy Thangavel were instrumental in my scientific training. I would like to acknowledge Jason Moore, Cathlyn Chan Jacob Elam, Anna Birg, Joseph Chapman, and Maria Dopico for their help as summer undergraduate research assistants.

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Most importantly, I would like to thank my family for all of the love and encouragement they have provided at every step.

ABSTRACT

Adenylyl cyclase (AC) catalyzes the formation of the ubiquitous second messenger cAMP. AC isoforms differ in their tissue distribution, cellular localization, regulation, and protein interactions, and most cells express multiple isoforms. We hypothesized that cAMP produced by different AC isoforms regulates unique cellular responses. Overexpression of individual isoforms had distinct effects on forskolin (Fsk)-induced expression of a number of known cAMP-responsive genes in human bronchial smooth muscle cells (BSMC) and human embryonic kidney cells (HEK-293). Most notable, in BSMC overexpression and activation of AC2 enhanced interleukin 6 (IL-6) expression, but overexpression of AC3 or AC6 had no effect. IL-6 production by BSMC was induced by Fsk and select G protein-coupled receptor (GPCR) agonists, though IL-6 levels did not directly correlate with intracellular cAMP levels. At low cAMP concentrations exchange protein directly activated by cAMP (Epac) predominated in mediating the IL-6 response, but at higher cAMP concentrations protein kinase A (PKA) assumed the larger role. IL-6 promoter mutations demonstrated that activator protein 1 (AP-1) and cAMP responsive element (CRE) transcription sites were required for cAMP mediated induction. Our findings indicate that AC2 participates in a cAMP-signaling compartment that specifically regulates IL-6 expression in BSMC and that other AC isoforms are excluded from this compartment.

TABLE OF CONTENTS

CHAPTER 1. INTRODUCTION	1
Bronchial Smooth Muscle and Asthma	1
GPCR and cAMP Signaling	2
β AR Agonists.....	3
cAMP Signaling Compartments	3
CHAPTER 2. METHODS	7
Materials	7
Cell Culture.....	7
AC Overexpression.....	7
PCR Array.....	8
qRT-PCR	8
ELISA	8
IL-6 Promoter Activity Assay.....	9
cAMP Assay	9
Data Analysis and Statistics.....	9
CHAPTER 3. cAMP-MEDIATED GENE REGULATION IS AC ISOFORM-DEPENDENT	10
Introduction.....	10
Results.....	11
A number of genes are differentially regulated by overexpression and activation of specific AC isoforms	11
Fsk-induced SST mRNA expression and protein production is specifically regulated by AC6 in BSMC	13
Fsk-induced IL-6 mRNA expression and protein production is specifically regulated by AC2 in BSMC	18
IL-6 basal expression and induction by Fsk differ in BSMC from an asthmatic donor	18
Discussion.....	24
Cell-type differences	24
IL-6 and asthma pathogenesis.....	26
AC isoform differences.....	26
CHAPTER 4. RECEPTOR MEDIATED IL-6 INDUCTION IN BSMC	27
Introduction.....	27
Results.....	28
Receptor-mediated induction of IL-6 is enhanced specifically by AC2.....	28
BSMC express several GPCR.....	31
Not all receptors are associated with induction of IL-6	31
IL-6 and cAMP levels are not directly correlated.....	36
Discussion.....	36

CHAPTER 5. DOWNSTREAM SIGNALING AND PROMOTER ELEMENTS ASSOCIATED WITH cAMP INDUCTION OF IL-6.....	39
Introduction.....	39
Results.....	40
PKA and Epac signaling induce IL-6 production	40
Inhibition of PKC, p38 MAPK, or PI3K does not block Fsk induction of IL-6.....	40
Activation of the IL-6 promoter by Fsk requires AP-1 and CRE sites	43
Discussion.....	43
CHAPTER 6. FINAL DISCUSSION AND FUTURE DIRECTIONS.....	47
AC Isoform-Specific Responses	47
Downstream Signaling Pathways	48
RNA Stabilization.....	48
Changes in Disease	50
Phosphoproteomics.....	51
AC Isoform-Specific Regulation of Other Genes.....	54
LIST OF REFERENCES.....	55
APPENDIX. PCR ARRAYS.....	70
VITA	89

LIST OF TABLES

Table 1-1.	Isoform-specific regulation of adenylyl cyclases	5
Table 4-1.	GPCR agonists and their targets in BSMC.....	32
Table A-1.	Fsk-induced gene regulation in AC overexpressing BSMC compared to control BSMC	70
Table A-2.	Fsk-induced gene regulation in AC overexpressing HEK compared to control HEK	77
Table A-3.	GPCR expression in BSMC	80

LIST OF FIGURES

Figure 3-1. Fsk-induced gene regulation in AC overexpressing BSMC compared to control BSMC	12
Figure 3-2. Fsk-induced gene regulation in AC overexpressing HEK-293 compared to control HEK-293	14
Figure 3-3. Time course of Fsk-induced SST mRNA expression	15
Figure 3-4. Fsk-induced SST mRNA expression in AC overexpressing BSMC	16
Figure 3-5. Fsk-induced SST protein production in AC overexpressing BSMC	17
Figure 3-6. Time course of Fsk-induced IL-6 mRNA expression	19
Figure 3-7. Fsk-induced IL-6 mRNA expression and protein production in AC overexpressing BSMC	20
Figure 3-8. Time course of Fsk-stimulated IL-6 protein production	21
Figure 3-9. Inhibition of Fsk-induced IL-6 expression with AC2-selective inhibitor	22
Figure 3-10. Inhibition of Fsk-induced IL-6 promoter activation with AC6-selective inhibitor	23
Figure 3-11. Fsk induction of IL-6 in normal and asthmatic BSMC	25
Figure 4-1. IL-6 mRNA expression in response to GPCR agonists	29
Figure 4-2. IL-6 protein production in response to GPCR agonists	30
Figure 4-3. cAMP and IL-6 protein production in response to $G\alpha_s$ -coupled receptor agonists	35
Figure 4-4. cAMP and IL-6 production in BSMC by GPCR agonists alone or with concurrent Fsk treatment	37
Figure 5-1. IL-6 protein production induced by cAMP analogs	41
Figure 5-2. IL-6 protein production by Fsk in the presence of PKA inhibitor	42
Figure 5-3. Fsk-induced IL-6 expression in the presence of kinase inhibitors	44
Figure 5-4. Promoter activity of wild type and mutant IL-6 promoter constructs	45
Figure 6-1. cAMP-mediated induction of IL-6 in BSMC	49

Figure 6-2. SILAC experimental setup.....	52
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LIST OF ABBREVIATIONS

βAR	β-adrenergic receptor
AC	Adenylyl cyclase
AKAP	A-kinase anchoring protein
AP-1	Activator protein 1
ASM	Airway smooth muscle
ATP	Adenosine triphosphate
ATPyS	Adenosine 5'-[γ-thio]triphosphate
AVP	Arginine vasopressin
BALF	Bronchoalveolar lavage fluid
BSMC	Bronchial smooth muscle cell
Buta	Butaprost
cAMP	Cyclic adenosine monophosphate
C/EBP	Ccaat-enhancer-binding protein
CGRP	Calcitonin gene related peptide
COX	Cyclooxygenase
CP	Crossing point
CRE	cAMP response element
Epac	Exchange protein directly activated by cAMP
EPR	Prostaglandin E receptor
Fsk	Forskolin
GPCR	G protein-coupled receptor
HEK-293	Human embryonic kidney 293A cell line
IκK	Inhibitor of κB kinase
IBMX	3-isobutyl-1-methylxanthine
IL-6	Interleukin 6
IP ₃	Inositol triphosphate
Iso	Isoproterenol
MAPK	Mitogen-activated protein kinase
NECA	5'-(N-Ethylcarboxamido)adenosine
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PDE	Phosphodiesterase
PGD ₂	Prostaglandin D ₂
PGE	Prostaglandin E
PI3K	Phosphatidylinositolide 3-kinases
PKA	Protein kinase A
PKC	Protein kinase C
SILAC	Stable isotope labeling by amino acids in cell culture
SST	Somatostatin

CHAPTER 1. INTRODUCTION

G protein-coupled receptors (GPCR) are one of the most utilized targets for therapeutic drugs, with up to 50% of pharmaceuticals acting at these receptors (Salon et al. 2011). Stimulation of a receptor results in generation of second messengers such as cyclic adenosine monophosphate (cAMP), inositol trisphosphate (IP₃), and diacylglycerol, and then signal transduction proceeds through a wide range of downstream effectors. GPCR regulate numerous physiological responses depending on the ligand, receptor, and cell-type. With few second messengers initiating a huge array of potential responses, cells must have mechanisms in place to direct the proper response upon agonist binding, however the mechanisms responsible are poorly understood. We are interested in how cells integrate and separate common signaling pathways and produce distinct responses. We hypothesize that signaling compartments are essential for directing specific cellular responses from a given GPCR. A better understanding of intracellular signaling compartments could allow for more specifically targeted therapeutics.

Bronchial Smooth Muscle and Asthma

Bronchial smooth muscle cells (BSMC) are critical in the pathogenesis and treatment of asthma. They possess secretory and contractile properties and are involved in the airway hyper-responsiveness, constriction, and remodeling that occur in asthma (Tliba and Panettieri 2009). In asthmatics there is an increase in both the size and number of airway smooth muscle cells (James et al. 2012). Asthmatic BSMC produce and secrete higher levels of inflammatory cytokines and extra cellular matrix proteins, contributing to inflammation and remodeling (Oliver et al. 2006; Johnson et al. 2004). It has been debated whether the pro-inflammatory environment of the asthmatic airway or changes to the cells themselves are responsible for alterations in the structure and function of airway smooth muscle (ASM) in asthma, and there is evidence that both play a role (Shore 2004). Bronchoalveolar lavage fluid (BALF) from allergen-challenged asthmatics causes increased proliferation of normal BSMC in culture, suggesting that the environment could be responsible for some of the phenotypic changes observed in asthma (Naureckas et al. 1999). A number of studies have also shown intrinsic differences in BSMC from asthmatic and nonasthmatic airways (Yeganeh et al. 2013). In vitro BSMC from asthmatic donors show greater contractility in response to histamine (Matsumoto et al. 2007). Genetic changes have also been observed that might explain phenotypic differences in BSMC of asthmatics (Oliver et al. 2006; Martin and Jo 2008; Roth et al. 2004).

GPCR signaling pathways, key to the pathogenesis and treatment of asthma, are also altered in BSMC of asthmatics. An imbalance of pro-contractile to pro-relaxant signaling occurs in asthma (Billington and Penn 2003). β -adrenergic receptor (β AR) agonists are less potent inducers of relaxation in carbachol contracted bronchial strips from asthmatic donors, suggesting reduced number or activity of the receptors (Goldie et

al. 1986). Polymorphisms in β_2 AR have been shown to enhance receptor down-regulation and may increase the propensity to develop certain types of asthma (Small et al. 2003; Turki et al. 1995). In addition to changes at the level of the receptor, phosphodiesterase (PDE) 4 expression and activity is increased in asthmatic BSMC, thus reducing cAMP signaling and further shifting the balance toward contraction (Trian et al. 2011). The shift in the balance of contractile to relaxant signaling may contribute to development and severity of asthma.

Airway constriction associated with asthma is treated with β AR agonists, which stimulate smooth muscle relaxation and bronchodilation largely through cAMP signaling pathways. Intermittent asthma can be treated at the time of exacerbation with short-acting β AR agonists for rapid bronchodilation. In moderate or severe asthma, in addition to short-acting β AR agonists as needed, long-acting β AR agonists are used in combination with inhaled corticosteroids as maintenance therapy (National Asthma and Prevention 2002). β AR agonists cause relaxation by activating cAMP signaling pathways and potassium channel activity, but they can also initiate several other responses, and some are detrimental to the asthmatic airway as discussed below.

GPCR and cAMP Signaling

GPCR are membrane-spanning proteins with extracellular ligand binding pockets and intracellular coupling to G-proteins. When a hormone, neurotransmitter, or drug binds to a GPCR on the cell surface, it initiates an intracellular signaling cascade. Ligand binding to the GPCR results in a conformational change of the receptor and interactions with G-proteins leading to GTP exchange and dissociation of the heterotrimeric G protein into G_α and $G_{\beta\gamma}$ subunits. G-proteins have particular effects depending on their subtype. $G_{\alpha s}$ stimulates and $G_{\alpha i}$ inhibits activity of adenylyl cyclase (AC), the enzyme that catalyzes the formation of cAMP from adenosine triphosphate (ATP). $G_{\alpha q}$ activates phospholipase C (PLC) that initiates pathways involved in phospholipid and Ca^{2+} signaling through IP_3 and diacylglycerol (Pierce et al. 2002). Each cell can express many GPCR, allowing it to respond to a variety of stimuli (Insel et al. 2012). In BSMC, GPCR signaling regulates cell cycle progression, contraction ($G_{\alpha q}$, $G_{\alpha i}$) and relaxation ($G_{\alpha s}$), and gene expression, among many other processes (Billington and Penn 2003).

Following $G_{\alpha s}$ stimulation, AC catalyze the formation of cAMP from ATP. cAMP regulates many cellular processes by activating protein kinase A (PKA) and exchange protein directly activated by cAMP (Epac) (Walsh et al. 1968; de Rooij et al. 1998). cAMP signaling is important in the treatment of asthma, because it causes relaxation of BSMC and dilation of the airway. Upon activation by cAMP, PKA phosphorylates several targets that lead to relaxation of smooth muscle cells (Knox and Tattersfield 1995). Calcium is the primary mediator of contraction, and cAMP signaling pathways oppose contractile pathways by regulating intracellular calcium concentrations and calcium sensitivity of contractile machinery. cAMP inhibits IP_3 -mediated Ca^{2+} release and facilitates Ca^{2+} uptake by the sarcoplasmic reticulum (Murthy 2001). PKA-

mediated phosphorylation of phospholamban reduces intracellular calcium by relieving phospholamban's inhibition of sarcoplasmic reticulum calcium pump ATPase channels (Prakash et al. 1997; Simmerman et al. 1986). Smooth muscle contraction occurs via myosin cross bridge cycling, and PKA plays a role in regulating the process. Phosphorylation of myosin light chain increases interactions with actin and ATPase activity and results in contraction. PKA phosphorylation promotes relaxation by decreasing the activity of myosin light chain kinase and increasing activity of myosin light chain phosphatase (Conti and Adelstein 1981; Pfitzer 2001; Wooldridge et al. 2004). G_{as} and PKA also facilitate β AR-mediated relaxation by activating potassium channels (Kume et al. 1994). The pro-relaxant actions of cAMP are taken advantage of in the treatment of asthma by β AR agonist therapies.

β AR Agonists

β AR agonists activate the cAMP signaling pathways described above and are used for bronchoprotection and as rescue treatment in asthma. The goal of β AR agonist therapy is to cause relaxation of smooth muscle and bronchodilation through G_{as} and cAMP signaling, but β AR agonists can also induce other signaling pathways that are not favorable in the asthmatic airway. Two main downsides of β AR agonists are changes in β AR coupling and desensitization. β_2 AR that initially couple to G_{as} can undergo G-switching upon exposure to agonist (Daaka et al. 1997b). Switching to G_{ai} coupling leads to activation of mitogen activated protein kinase (MAPK) cascades that induce expression of pro-inflammatory genes and can worsen asthma (Pelaia et al. 2005). β_2 AR can also exhibit atypical coupling to G_{aq} , which increases Ca^{2+} and contractile responses (Anderson 2006). Chronic β AR agonist exposure leads to tolerance and desensitization (Cooper et al. 2011; Benovic et al. 1985). Several mechanisms can play a role in desensitization and downregulation of β AR following stimulation, which result in decreased responsiveness to therapy over time (Lohse et al. 1990; Giembycz and Newton 2006). Phosphorylation by PKA reduces receptor affinity for G_{as} and increases affinity for G_{ai} (G switching) (Daaka et al. 1997a). Phosphorylation by G protein coupled-receptor kinase (GRK) recruits β arrestin and facilitates internalization of the receptor via clathrin-coated pits (Benovic et al. 1988). Due to altered coupling and responsiveness of β AR, treatment with β AR agonists alone can lead to increased hyperresponsiveness and inflammation, reduced bronchodilation, and even increased risk of morbidity and mortality (Cheung et al. 1992; Johnston and Edwards 2009; Strandberg et al. 2007). In the future AC may represent a better therapeutic target with fewer unintended effects, since only cAMP pathways would be activated without G_{ai} or G_{aq} activation. Approaches targeting AC also have the potential to create larger changes in cAMP, since AC is the limiting component in β AR-stimulated cAMP production (Ostrom et al. 2000b).

cAMP Signaling Compartments

cAMP is ubiquitous and mediates many responses in BSMC. It would not be practical for cAMP to freely float around the cell activating numerous pathways in

response to each stimulus, and in fact steady state concentrations would likely not be high enough to activate PKA (Rich et al. 2000). Compartmentalized cAMP signaling allows specific responses to stimuli. The first examples of compartmentation of cAMP signals were described in myocardium where it was observed that of two agonists, epinephrine and prostaglandin E₁ (PGE₁) that act through cAMP and PKA, only epinephrine stimulated contractile force and glycogen phosphorylase activity (Keely 1977, 1979). Homogenate fractionation showed that these stimuli activated PKA in different compartments (Buxton and Brunton 1983). Compartmentation involves components both upstream and downstream of cAMP production. There are nine AC isoforms, which differ in their tissue distribution, regulation (**Table 1-1**), cellular localization, and associations with other proteins. We hypothesize that the differences among isoforms help them define unique cAMP compartments and lead to AC isoform-specific cellular and physiological responses.

Several components in addition to AC contribute to establishment of cAMP signaling compartments. PDE hydrolyze cAMP, limiting its diffusion from the site of production (Mika et al. 2012). A-kinase anchoring protein (AKAP) scaffolding molecules bring together multiple signaling molecules in complexes (Smith et al. 2006). Over 50 AKAP have been described, and like AC they have unique tissue distribution, intracellular localization, and interactions (Dessauer 2009). In addition to prearranging signal cascades, AKAP bind PDE and regulators of AC, keeping cAMP near the site of production. AKAP and direct complexes containing AC allow for rapid and precise signaling to generate specific cellular responses downstream of a given stimulus (Ostrom et al. 2012).

Although specific physiological responses following activation of different G_{as} receptors have been known since the 1970's, AC isoform-specific responses have only recently been described. AC5 and AC6, though similar in their structure and regulation, have been shown to have unique roles in the heart through overexpression and knockout studies (Pierre et al. 2009). In cardiomyopathy models, AC6 overexpression increases survival and heart function (Roth et al. 2002). Conversely, it is knockdown of AC5 that is cardioprotective (Vatner et al. 2009). Differences in the localization and associations of AC5 and AC6 appear to drive the isoform-specific effects in cardiomyocytes. AC6 is located outside T-tubules where it colocalizes with β_2 AR (Timofeyev et al. 2013). AC6 activation in response to β AR agonist results in increased L-type calcium current (Timofeyev et al. 2013). AC5 on the other hand is localized to T-tubules where it is in a complex containing PDE, and cAMP diffusion is tightly constrained and unable to alter calcium currents (Timofeyev et al. 2013). Interestingly, even a catalytically inactive AC6 mutant has been shown to have beneficial effects when overexpressed in mice with cardiomyopathy, likely by regulating Akt signaling through direct interactions with PH domain and Leucine rich repeat Protein Phosphatase 2 (Gao et al. 2009; Gao et al. 2011). The benefits of AC6 signaling in failing hearts are so promising that clinical trials are underway utilizing AC6 gene transfer in patients with congestive heart failure (Tang et al. 2012). Other examples of AC isoform-specific regulation of cellular responses have been described in vascular and airway smooth muscle. In vascular smooth muscle, AC1 mediates proliferation, while AC6 has no impact on proliferation but is involved in

Table 1-1. Isoform-specific regulation of adenylyl cyclases

Regulator	Effect	AC subtype	References
G protein			
Gs	Stimulation	All Isoforms	(Iyengar 1993)
Gi	Inhibition	AC1, AC5, AC6; not AC2	(Taussig et al. 1993a) (Federman et al. 1992) (Chen and Iyengar 1993) (Taussig et al. 1994)
Gβγ	Inhibition	AC1, AC5, AC6	(Taussig et al. 1993b) (Bayewitch et al. 1998)
	Stimulation	AC2, AC4 Conditional AC5, AC6	(Tang and Gilman 1991) (Gao and Gilman 1991) (Gao et al. 2007)
Forskolin	Stimulation	All Isoforms (including AC9)	(Onda et al. 2001) (Premont et al. 1996) (Cumbay and Watts 2004)
Calcium/Calmodulin			
Ca ²⁺ /CaM	Stimulation	AC1, AC3, AC8	(Tang et al. 1991) (Choi et al. 1992) (Cali et al. 1994)
Ca ²⁺	Inhibition	AC5, AC6	(Yoshimura and Cooper 1992) (Katsushika et al. 1992)
Kinase Regulation			
PKC	Stimulation	AC1, AC2, AC3, AC5, AC7	(Jacobowitz et al. 1993) (Jacobowitz and Iyengar 1994) (Bol et al. 1997) (Kawabe et al. 1994) (Watson et al. 1994)
	Inhibition	AC6	(Lai et al. 1997)
PKA	Inhibition	AC5, AC6	(Iwami et al. 1995) (Chen et al. 1997)
CaM Kinase	Inhibition	AC1, AC3	(Wayman et al. 1996) (Wei et al. 1996)
Raf kinase	Stimulation	AC2, AC5, AC6	(Ding et al. 2004)

Reprinted with permission. Ostrom RS, Bogard AS, Gros R, Feldman RD (2012) Choreographing the adenylyl cyclase signalosome: sorting out the partners and the steps. *Naunyn Schmiedeberg's Arch Pharmacol* 385 (1):5-12.
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With knowledge of differences among AC isoforms and compartmentalization of cAMP signaling, we hypothesized that BSMC possess AC isoform-specific regulation of cellular processes. We examined regulation of cAMP-responsive genes in BSMC overexpressing individual AC isoforms and uncovered a number of genes that are regulated in an isoform-specific manner. AC2-derived cAMP selectively regulates expression of the pro-inflammatory cytokine interleukin 6 (IL-6), a key contributor to the pathogenesis of asthma. cAMP induces IL-6 production, but the levels of cAMP and IL-6 are not directly correlated. AC2's unique localization, regulation and interactions allow it to activate PKA and Epac in a signaling compartment associated with induction of IL-6. AC6-derived cAMP is tied to regulation of other genes, but PDE limit this cAMP pool from activating components involved in IL-6 induction. The cAMP compartments and signaling pathways responsible for stimulation of IL-6 transcription appear to be altered in asthma. β AR agonists currently represent the best therapy for treatment of bronchoconstriction in asthma, but some of the signaling pathways activated by β AR worsen inflammation and constriction. Targeting AC directly instead of through β AR and $G_{\alpha s}$ would stimulate cAMP production without activation of $G_{\alpha i}$ and $G_{\alpha q}$ and could result in greater cAMP production. We show that cAMP can also mediate undesirable responses in BSMC, in particular induction of IL-6. Since cAMP signaling is highly compartmentalized, therapeutics targeting AC6 may stimulate ASM relaxation and bronchodilation without inducing IL-6 production. A greater knowledge of cAMP signaling compartments could allow highly targeted treatment for many diseases, including asthma.

CHAPTER 2. METHODS

Materials

Forskolin (Fsk), isoproterenol (Iso), 3-isobutyl-1-methylxanthine (IBMX), Adenosine 5'-[γ -thio]triphosphate (ATP γ S), Arginine vasopressin (AVP), prostaglandin D₂ (PGD₂), glucagon, 5'-(N-Ethylcarboxamido)adenosine (NECA), calcitonin gene related peptide (CGRP), and substance P were purchased from Sigma; 8-Br-cAMP, 8CPT-2Me-cAMP, GF 109203X, SB 202190, ($\pm$)-SKF-83566, SQ22,536 and wortmannin from Tocris, and butaprost (buta) from Cayman.

Wild-type and mutant IL-6 promoter luciferase constructs were purchased from the Belgian Coordinated Collections of Micro-organisms/LMBP; LMBP acquisition numbers 4495, 4496, 4498, 4499, 4500 (Vanden Berghe et al. 1998; Plaisance et al. 1997). Luciferase constructs contain 1168 bp of the human IL-6 promoter (wild-type or with point mutations in specific promoter elements) upstream of LUCm in pGL3-Basic vector. Binding sites were altered by site-directed mutagenesis to prevent transcription factor binding at the following promoter elements: 3' or 5' activator protein 1 (AP-1) site, cAMP response element (CRE), CCAAT-enhancer-binding proteins (C/EBP), or nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B) sites.

Cell Culture

Human BSMC purchased from Lonza were grown in smooth muscle basal medium supplemented with the SmGM-2 bullet kit (5% fetal bovine serum, 0.1% insulin, 0.1% human epidermal growth factor, 0.2% human fibroblast growth factor- β , and gentamicin sulfate/amphotericin B; Lonza). Cells were kept at 5% CO₂ and 37° C. Experiments were performed on cells from passage 5-13.

Human embryonic kidney 293A cell line (HEK-293) purchased from Invitrogen were grown in Dulbecco's Modified Eagle's medium (DMEM) (high glucose) with 10% fetal bovine serum, 0.1 mM MEM Non-Essential Amino Acids, 2 mM L-glutamine, and 1% Pen-Strep. Cells were kept at 5% CO₂ and 37° C.

AC Overexpression

Adenoviral constructs expressing rat AC2, rat AC3, mouse AC6, or lacZ (control) cDNA were used for AC overexpression studies in BSMC. The titer of AC virus was chosen to give similar global cAMP levels in response to 1 μ M Fsk. Cells were infected 18-24 h before treatment.

Plasmids encoding rat AC2, human AC6, or the empty pEGFP-n1 vector were used for overexpression studies in HEK-293. Cells were transfected with Cal-Phos

calcium phosphate transfection kits (Clontech) 24 h after plating. Cells were transfected with 3 µg plasmid per 60 mm dish. For polymerase chain reaction (PCR) arrays Fsk was added 24 h after transfection and RNA was isolated 24 h later as described for PCR array below.

PCR Array

Following treatment cells were lysed and RNA was isolated using RNeasy kit with on-column DNase step (Qiagen). RNA purity and yield were determined with Nanodrop spectrophotometer. 1 µg RNA was reverse transcribed using RT² first strand kit (SABioscience). cDNA from a single sample was combined with RT² SYBR Green Master Mix (SABioscience) and distributed among wells of a multiwell plate containing gene specific and control primers on Human cAMP/Ca²⁺ PathwayFinder RT² Profiler™ or Human G Protein Coupled Receptors 384HT RT² Profiler™ PCR Array (SABioscience). The crossing point (CP) was determined by the second derivative maximum algorithm on Roche Lightcycler 480, and fold change was calculated by $\Delta\Delta CP$ method. Melt curve analysis was used to exclude any gene whose CP may have been artificially reduced by multiple amplification products.

qRT-PCR

For quantitative reverse transcriptase PCR (qRT-PCR) RNA was isolated as described for PCR arrays. 1 µg RNA was reverse transcribed using Transcriptor First Strand cDNA Synthesis Kit (Roche) and oligo (dT)¹⁸ primer. PCR was carried out on Roche Lightcycler 480: 10 min 95° C followed by 45 cycles of 95° C for 10 s, 55° C for 30 s, 72° C for 6 s. Amplification was detected by SYBR green (KAPA) and single PCR products were confirmed by melt curve analysis. Fold regulation was calculated by $\Delta\Delta CP$ method with normalization to RPL13A housekeeping gene. The following gene-specific primer sets were used:

IL-6 Forward: GAC AGC CACTCA CCT CTT CA

IL-6 Reverse: AGT GCCTCT TTG CTG CTT TC

SST Forward: TCT GAA CCC AAC CAG AAG GAG AAT

SST Reverse: GCT CAA GCC TCA TTT CAT CCT GCT

ELISA

IL-6 in cell culture medium was measured by sandwich ELISA according to manufacture's instructions (IL-6: eBioscience or BD bioscience, SST: Phoenix). ELISA were read on Synergy HT (Biotek) plate reader.

IL-6 Promoter Activity Assay

BSMC were plated 30,000 cells per well in 12-well plates. 18-24 h after plating, each well was transfected with 1.8 μ g plasmid expressing the human IL-6 promoter driving firefly luciferase expression. Cells were co-transfected with 0.4 μ g *Renilla* luciferase intended as an internal control constitutively expressed via the thymidine kinase promoter. However, Fsk treatment affected *Renilla* luciferase expression, so it could not be used for normalization in our studies. All transfections used the CalPhos (Clontech) calcium phosphate kit. Experiments were begun 48 h post-transfection. Following treatment with the indicated drugs for 6 h, cells were scraped in 250 μ L passive lysis buffer and assayed using the Dual Luciferase Reporter Assay System (Promega). Luminescence was measured with a TD-20/20 luminometer (Turner Designs).

cAMP Assay

Cells were washed three times with serum- and NaHCO₃-free Dulbecco's Modified Eagle's medium (DMEM) supplemented with 20 mM HEPES, pH 7.4 (DMEH). After equilibration at 37°C for 30 min, cells were pretreated with 0.2 mM IBMX, a broadly specific PDE inhibitor, then exposed to the indicated drug for 10 min. Assay medium was aspirated and 150 μ L 5% trichloroacetic acid was added to each well to terminate the reaction. cAMP content of the lysis buffer extract was quantified using the cAMP EIA Kit (Cayman Chemical) using the manufacturer's acetylation protocol.

Data Analysis and Statistics

Data are presented as the mean $\pm$ SEM. Statistical comparisons (*t* tests and one-way analysis of variance) were performed and graphics were generated using GraphPad Prism 5.0f (GraphPad Software Inc., San Diego, CA).

CHAPTER 3. cAMP-MEDIATED GENE REGULATION IS AC ISOFORM-DEPENDENT

Introduction

BSMC predominantly express AC isoforms 2, 4 and 6 (Bogard et al. 2011). These isoforms differ in their regulation (**Table 1-1**), localization, and interactions with other proteins, giving them the potential to signal in unique ways and control distinct responses even when expressed in the same cell. AC isoforms are divided into 4 groups based on their structure and features of their regulation. Group I consists of AC1, AC3, and AC8; group II: AC2, AC4, and AC7; group III: AC5 and AC6; and group IV: AC9 (Patel et al. 2001). In BSMC, AC6 is localized to lipid rafts where it colocalizes with β_2 AR (Bogard et al. 2011). AC2 and AC4 are excluded from lipid rafts and colocalize with prostaglandin E₂ receptors (EP₂R) (Bogard et al. 2011). BSMC express several AKAP scaffolding molecules, which arrange signaling complexes and interact with AC in an isoform-specific manner (Dessauer 2009; Horvat et al. 2012). AC2 is known to interact with multiple AKAP including mAKAP, Yotiao/AKAP9, and AKAP79/150, which bring cAMP produced by AC2 in close proximity to prearranged signaling cascades and regulators such as PDE and phosphatases (Piggott et al. 2008; Kapiloff et al. 2009; Scott et al. 2013). AC6 is also associated with AKAP, and it has been shown to interact directly with AKAP79 (Efendiev et al. 2010).

AC is the limiting factor in determining the maximal effect following G_{as}-coupled receptor stimulation (Ostrom et al. 2000b; Gao et al. 1998). Overexpression of AC enhances responses to G_{as}-coupled receptor stimulation. In BSMC we use adenoviral overexpression of AC and subtractive analysis to study responses mediated by individual isoforms. BSMC have low transfection efficiency, but adenoviral infection results in a high percentage of cells overexpressing protein of interest. β galactosidase assays confirmed over 90% of BSMC are positive for lacZ when infected with lacZ encoding adenovirus (Bogard et al. 2011). Knockdown of AC isoforms with siRNA would be an alternative approach and is appealing, since the individual isoforms that are left would be expressed at endogenous levels. However, a high degree of knockdown would be unlikely due to the long half-life of AC, and it would be particularly difficult in poorly transfected cells such as BSMC. We also used newly characterized AC inhibitors selective for particular AC isoforms. A number of pharmacological inhibitors have been used to study AC isoforms, but questions were raised about claims of presumed isoform selectivity of these inhibitors. Two recent studies sought to better describe the effect of inhibitors on particular AC isoforms. Though previously described as an AC5-selective inhibitor, SQ22,536 inhibits AC5 and AC6 equally. The IC₅₀ for SQ22,536 at AC6 is 5.8 μ M, and its IC₅₀ at AC2 is 210 μ M (Brand et al. 2013). Conley et. al. screened over 700 compounds and identified SKF-83566 as an AC2-selective inhibitor (2013). We hypothesized that differences among AC isoforms and compartmentation of cAMP signaling allows AC isoform-specific regulation of gene expression. We used PCR arrays and isoform-specific AC inhibitors to show that some cAMP-responsive genes are differentially regulated by individual AC isoforms in BSMC.

Results

A number of genes are differentially regulated by overexpression and activation of specific AC isoforms

We tested the hypothesis that cAMP produced by a particular AC isoform has unique effects on gene expression by using PCR arrays to examine 84 cAMP- and Ca^{2+} -sensitive genes. PCR arrays allow simultaneous analysis of multiple genes by utilizing specially designed primer pairs such that all have been optimized for amplification of their specific target under identical conditions. Control or AC overexpressing cells were treated with 1 μM Fsk for 24 h before RNA was isolated and reverse transcribed. The resulting cDNA was diluted in SYBR green master mix and distributed among the wells of the PCR array, each with a primer pair specific to a particular gene or control.

Expression of each gene in AC overexpressing cells treated with Fsk was compared to Fsk-treated control cells. Control cells were transfected with empty pEGFP-n1 vector or treated with an adenovirus expressing the lacZ gene. Fold change was calculated by the $\Delta\Delta\text{CP}$ method with respect to lacZ Fsk-treated cells. A change in expression of 3-fold or greater was the cutoff we used to highlight genes that were upregulated or downregulated upon AC overexpression. For genes that displayed a substantial change with AC overexpression, we examined whether there were differences in gene regulation based on overexpression of individual isoforms. Changes in RNA levels smaller than 3-fold might be physiologically significant, but we choose to focus on the larger responses in this study. Although Fsk treatment altered the expression of many of the genes compared to vehicle in control BSMC, we focused on genes that were differentially regulated with overexpression of specific AC isoforms.

BSMC

Adenoviral vectors were used to overexpress AC2, AC3, AC6, or lacZ (control) in BSMC. AC2 and AC6 are natively expressed in distinct membrane microdomains in BSMC and AC3 was included as a representative group I isoform. AC overexpression altered the Fsk-induced expression of 13 of the cAMP-sensitive genes tested by at least 3-fold in BSMC (**Table A-1**). The genes of greatest interest were those that were differentially regulated when different AC were overexpressed. Overexpression of AC3 did not produce a 3-fold or greater change in any of the genes tested; however, AC2 and AC6 had distinct effects on expression of a number of genes (**Figure 3-1**). AC2 and AC6 overexpression could enhance or attenuate Fsk induction and had differing effects depending on the AC isoform and the gene. A subset of genes was chosen to demonstrate the diversity of responses with different AC isoforms overexpressed, and results are graphed as fold change with respect to lacZ to illustrate the effect of the individual AC being overexpressed (**Figure 3-1**). Fsk-mediated expression of amphiregulin (AREG) was augmented to a greater extent by AC6 overexpression than AC2. AC2 overexpression reduced the expression of secretogranin II (SCG2), while

Figure 3-1. Fsk-induced gene regulation in AC overexpressing BSMC compared to control BSMC

RT²q PCR arrays (SA Biosciences) were used to measure mRNA levels following 24 h treatment with 1 μ M Fsk. mRNA levels of AC overexpressing, Fsk-treated cells are expressed as fold change with respect to lacZ (control), Fsk- treated cells. Amphiregulin (AREG), Secretogranin II (SCG2), Cyclin D1 (CCND1), Interleukin 6 (IL-6), Somatostatin (SST). Dashed line represents no change with respect to lacZ. Data are presented as fold change with respect to lacZ, mean $\pm$ SEM, n=3.

AC6 overexpression reduced the expression of cyclin D1 (CCND1). Fsk-mediated expression of Interleukin 6 (IL-6) was enhanced only by AC2 overexpression and somatostatin (SST) was enhanced only by AC6 overexpression (**Figure 3-1**). IL-6 and SST were targets for further study due to their clear AC isoform-dependent patterns of gene regulation in BSMC.

HEK-293

After finding that gene expression can be regulated differently by AC isoforms in BSMC, HEK-293 cells were tested to determine whether this is a generalizable phenomenon or unique to BSMC. HEK-293 cells were transfected with AC2, AC6, or pEGFP-n1 (control) vectors then exposed to 1 μ M Fsk for 24 h. As in BSMC, expression of some cAMP-sensitive genes was altered by AC overexpression. Different genes were impacted by AC overexpression in the different cell types, and in HEK-293 the effects of AC overexpression tended to be smaller (**Figure 3-2** and **Table A-2**). As in BSMC, AC overexpression could enhance or reduce Fsk-induced RNA expression in HEK-293 in an isoform-specific manner. Calponin 1 (CNN1) RNA levels were reduced by AC6 overexpression, but not AC2. Gastric inhibitory polypeptide receptor (GIPR) expression was enhanced only by AC2 overexpression and tumor necrosis factor (TNF) expression was enhanced only by AC6 overexpression (**Figure 3-2**).

Fsk-induced SST mRNA expression and protein production is specifically regulated by AC6 in BSMC

In the PCR array, overexpression of AC6 enhanced Fsk-induced SST mRNA expression by 9.73 fold in BSMC, while AC2 overexpression had no effect on SST expression (**Figure 3-1**). 24 h was a reasonable starting point for measuring Fsk-stimulated expression of many different genes in the array, but once SST and IL-6 emerged as genes of interest with specific regulation by different AC isoforms, time course studies were completed to determine the optimal treatment time for further experiments. SST mRNA peaked at 3 h in response to 1 μ M Fsk (**Figure 3-3**).

One μ M Fsk resulted in a particularly robust induction of the SST gene, so 0.3 μ M Fsk treatments were used in subsequent studies to avoid a saturation of the response that might prevent noticeable enhancement by AC overexpression. Exposure to 0.3 μ M Fsk for 3 h caused a 26.68-fold increase in SST mRNA compared to vehicle in control cells (**Figure 3-4**). With overexpression of AC6, the effect of Fsk was enhanced and SST mRNA was 67.48-fold above vehicle. AC2 overexpression did not alter SST when compared to control cells with native AC expression (25.74-fold over vehicle).

To determine if the increased SST mRNA in AC6 overexpressing cells results in increased protein, we used ELISA to measure SST in cell culture medium. As early as 3 h the effect of AC6 overexpression was observed as increased SST protein (**Figure 3-5**). AC6 overexpression more than doubled Fsk-induced SST production by

Figure 3-2. Fsk-induced gene regulation in AC overexpressing HEK-293 compared to control HEK-293

RT²q PCR arrays (SA Biosciences) were used to measure mRNA levels following 24 h treatment with 1 μ M Fsk. mRNA levels of AC overexpressing, Fsk-treated cells are expressed as fold change with respect to empty pEGFP-n1 vector (control), Fsk- treated. Calponin 1 (CNN1), Gastric inhibitory polypeptide receptor (GIPR), Tumor necrosis factor (TNF). Dashed line represents no change with respect to empty vector. Data are presented as fold change with respect to empty vector, n=1.

Figure 3-3. Time course of Fsk-induced SST mRNA expression

SST mRNA expression was measured by qRT-PCR in AC6 overexpressing cells exposed to 1 μ M Fsk for the indicated time. SST mRNA levels are expressed with respect to lacZ basal, n=1.

Figure 3-4. Fsk-induced SST mRNA expression in AC overexpressing BSMC
qRT-PCR was used to determine SST RNA expression in BSMC treated with 0.3 μ M Fsk for 3 h. Data are presented as fold change with respect to lacZ basal, mean $\pm$ SEM, n=3.

Figure 3-5. Fsk-induced SST protein production in AC overexpressing BSMC
ELISA were used to measure SST secreted into culture medium by BSMC treated with 0.3 μM Fsk for 3 h. Data are presented as pg/mL SST. n=1-2.

BSMC in 3 h, from 50.29 pg/mL to 111.69 pg/mL. AC2 overexpression did not enhance SST production (**Figure 3-5**).

Fsk-induced IL-6 mRNA expression and protein production is specifically regulated by AC2 in BSMC

AC2 overexpression clearly enhanced Fsk induction of IL-6 in the PCR array, but AC6 overexpression had no effect. In control BSMC, time course studies showed that IL-6 mRNA peaked at 1 h in response to 1 μ M Fsk (**Figure 3-6**). At 1 h, 1 μ M Fsk increased IL-6 mRNA in control cells by 8.16-fold (**Figure 3-7A**). The enhancement of IL-6 mRNA levels by AC2 overexpression was less pronounced at 1 h (**Figure 3-7A**) than 24 h (**Figure 3-1**), but since Fsk-stimulated IL-6 mRNA levels peaked at 1 h, this time point was used in all subsequent studies. AC2 overexpression resulted in a near doubling of IL-6 mRNA compared to control cells also exposed to 1 μ M Fsk for 1 h. AC6 did not alter Fsk-induced IL-6 expression compared to control cells (**Figure 3-7A**).

To determine if the increased IL-6 mRNA in AC2 overexpressing cells translated to increased protein, IL-6 protein secreted into culture medium was measured by ELISA. 1 μ M Fsk resulted in IL-6 protein production that increased sharply between 10 and 24 h then continued to accumulate over time (**Figure 3-8**). 24 h was chosen as the time point for all other studies measuring IL-6 protein. Unlike mRNA, where Fsk induced a 8.16-fold increase over vehicle in control cells, IL-6 protein levels were only slightly increased by Fsk (**Figure 3-7B**). In control cells 24 h Fsk treatment increased IL-6 by 11% (from 594 pg/mL in vehicle-treated cells to 668 pg/mL in Fsk-treated). AC2 overexpression enhanced Fsk-induced IL-6 protein production to 942 pg/mL. Overexpression of AC6 did not increase IL-6 over control cells, and in fact IL-6 was slightly lower in AC6 overexpressing cells (652 pg/mL) compared to control BSMC with native AC expression (**Figure 3-7B**).

Recent studies characterizing AC inhibitors have identified SKF-83566 as AC2-selective and SQ22,536 as AC5/6-selective (Conley et al. 2013; Brand et al. 2013). We used these inhibitors as another approach to investigate the AC2-specific induction of IL-6. We pretreated BSMC with each of these inhibitors prior to 1 μ M Fsk treatment. Pretreatment with 30 μ M SKF-83566 for 20 min reduced Fsk induction of IL-6 mRNA by 48.21% (**Figure 3-9**). The effect of SQ22,536 pretreatment on IL-6 promoter activity was measured by luciferase assay. SQ22,536 did not significantly reduce Fsk-induced IL-6 promoter activity when the inhibitor was added at 1, 3, or 10 μ M for 30 min prior to Fsk treatment (**Figure 3-10**).

IL-6 basal expression and induction by Fsk differ in BSMC from an asthmatic donor

IL-6 levels in BALF and serum are increased in patients with asthma, and BSMC could be one contributor to raised IL-6 levels (Rincon and Irvin 2012). We

Figure 3-6. Time course of Fsk-induced IL-6 mRNA expression

qRT-PCR was used to measure IL-6 mRNA expression in control BSMC exposed to 1 μ M Fsk for the indicated time. Data are expressed as the inverse of the CP, mean $\pm$ SEM, n=2-3.

Figure 3-7. Fsk-induced IL-6 mRNA expression and protein production in AC overexpressing BSMC

(A) qRT-PCR was used to detect IL-6 mRNA levels in BSMC exposed to 1 μ M Fsk for 1 h. mRNA levels of Fsk-treated cells are expressed as fold change with respect to lacZ basal, mean $\pm$ SEM, n=3. Dashed line represents no change with respect to lacZ basal. * indicates $p < .05$ compared to lacZ. (B) ELISA were used to measure IL-6 secreted into culture medium by BSMC treated with 1 μ M Fsk for 24 h. Data are presented % change with respect to lacZ basal, mean $\pm$ SEM, n=3. * indicates $p < .05$ compared to lacZ basal.

Figure 3-8. Time course of Fsk-stimulated IL-6 protein production

ELISA were used to measure IL-6 protein in cell culture medium following exposure to 1 μ M Fsk for the indicated amount of time. Mean $\pm$ SEM, n=1-2.

Figure 3-9. Inhibition of Fsk-induced IL-6 expression with AC2-selective inhibitor

qRT-PCR was used to determine IL-6 mRNA levels in BSMC pretreated with 30 μ M SKF-83566 or vehicle for 20 min prior to 1 h, 1 μ M Fsk treatment. mRNA levels are expressed relative to vehicle treated, mean $\pm$ SEM, n=4. Dashed line represents no change with respect to vehicle. * indicates $p < .05$ compared to Fsk alone.

Figure 3-10. Inhibition of Fsk-induced IL-6 promoter activation with AC6-selective inhibitor

Luciferase assays were used to determine IL-6 promoter activation in BSMC pretreated with the indicated concentration of SQ22,536 or vehicle for 30 min prior to 6 h, 1 μ M Fsk treatment. Luminescence is expressed relative to vehicle treated, mean $\pm$ SEM, n=4.

wondered if cAMP-mediated IL-6 induction differed in BSMC from an asthmatic donor compared to a non-asthmatic donor. Cells from different donors were cultured in identical growth medium with the intent to uncover any intrinsic changes in IL-6 induction pathways in asthmatic BSMC. BSMC from an asthmatic donor had basal levels of IL-6 mRNA that were 3-times higher than BSMC from a non-asthmatic donor (**Figure 3-11**). When BSMC from the asthmatic donor were treated with 1 μ M Fsk, there was no increase in IL-6 mRNA compared to those cells untreated. In non-asthmatic BSMC treated at the same time, Fsk treatment caused a 2.6-fold increase in IL-6 mRNA levels. IL-6 expression is increased in asthmatic BSMC at rest, and AC activation by Fsk is unable to further stimulate IL-6 transcription as it does in healthy BSMC.

Discussion

A number of genes that are differentially regulated by individual AC isoforms in BSMC and HEK-293 were identified by PCR array (**Figures 3-1** and **3-2**). In BSMC IL-6 is selectively regulated by AC2 and SST is selectively regulated by AC6. AC isoform-specific enhancement of the induction of these genes results in increased protein production (**Figures 3-5** and **3-7B**). Basal expression of IL-6 is increased in asthmatic BSMC, and induction by Fsk is impaired (**Figure 3-11**).

Cell-type differences

BSMC are highly differentiated cells with unique physiological roles. To confirm that AC isoform-specific regulation of gene expression is a generalizable phenomenon, we also tested the less well differentiated HEK-293 cells. Both cell types have cAMP regulated genes and genes that are differentially regulated when specific AC isoforms are overexpressed (**Figures 3-1** and **3-2**). Interestingly, the AC isoforms that are predominately expressed in these cell types are similar, but the degree of compartmentation differs greatly. It is not surprising that cAMP stimulates different genes in cell types with distinct physiological roles.

One methodological difference in these studies is the mode of AC overexpression. BSMC have poor transfection efficiency, whether using lipofectamine or calcium phosphate transfection methods. Therefore adenoviral vectors were used to overexpress proteins of interest in BSMC. β galactosidase staining indicates lacZ expression in greater than 90% of BSMC following adenoviral mediated gene transfer (Bogard et al. 2011). HEK-293 efficiently take up plasmids when transfected, but adenoviral overexpression cannot be used in HEK-293. They cannot be infected with adenovirus to overexpress AC because they are packaging cells that contain the E1 and E3 adenoviral genes, which allow adenoviral replication and lysing of the cells.

Figure 3-11. Fsk induction of IL-6 in normal and asthmatic BSMC
qRT-PCR was used to detect mRNA levels in BSMC exposed to 1 μ M Fsk for 1 h. mRNA levels are expressed as fold change with respect to normal basal, mean $\pm$ SEM, n=3.

IL-6 and asthma pathogenesis

The finding that AC2 specifically regulates expression of the pro-inflammatory cytokine IL-6 in BSMC is of particular interest due to its role in the pathogenesis of asthma. IL-6 is increased in the serum and BALF of asthmatic patients and is inversely correlated with lung function (Neveu et al. ; Morjaria et al. 2011). IL-6 is more than just a marker of inflammation in asthma. It can be secreted by structural cells independent of inflammation and plays a role in the development and worsening of pathological changes associated with asthma (Rincon and Irvin 2012). IL-6 has many effects in the airway. The main targets of IL-6 are immune cells, but soluble IL-6 receptors and trans-signaling allow IL-6 to mediate responses in most cell-types (Rose-John et al. 2006). IL-6 encourages the differentiation of CD4⁺ T cells to Th2 cells and inhibits maturation of Th1 cells (Diehl and Rincon 2002). This bias toward Th2 is likely involved in many pathological features of asthma including inflammation, mucus secretion, and airway hyper-responsiveness (Georas et al. 2005). IL-6 increases airway resistance and causes smooth muscle hypertrophy and hyperplasia in rodents (De et al. 1995; Rubini 2010). IL-6 is obviously important in asthma pathology, and treatment with β AR agonists may be increasing IL-6 production through AC2-mediated signaling. A better understanding of the cAMP signaling compartments associated with regulating IL-6 expression could allow for more precisely targeted asthma therapies in the future.

We found that IL-6 expression was increased in BSMC from an asthmatic donor compared to cells from a healthy donor under basal conditions. BSMC from the asthmatic donor did not respond to Fsk with increased IL-6 expression as the normal BSMC did. The lack of Fsk response could be explained by the high basal IL-6 expression. If the pathways responsible for induction of IL-6 are constitutively active in asthmatic BSMC, Fsk may not have a further effect. **Figure 3-11** represents data gathered from multiple treatments of cells isolated from 2 donors. The data here do not allow us to draw conclusions about difference between asthmatic and non-asthmatic BSMC, because they are only from a single asthmatic and a single non-asthmatic donor. IL-6 induction by cAMP signaling in BSMC from asthmatic and non-asthmatic donors should be repeated with cells from additional donors before conclusions can be made.

AC isoform differences

We show that genes can be regulated differently by individual AC isoforms. In BSMC AC2, AC4, and AC6 are endogenously expressed, and we have shown that AC2 and AC6 have distinct effects on expression of various genes when they are overexpressed and activated in BSMC. AC3 was included in our study to represent group I AC, and it was not associated with changes in Fsk-mediated gene regulation compared to control BSMC. With IL-6 found to be specifically regulated by AC2-derived cAMP, the rest of this project focused on uncovering the signaling pathways involved to determine how AC2- but not AC6-derived cAMP is associated with induction of IL-6.

CHAPTER 4. RECEPTOR MEDIATED IL-6 INDUCTION IN BSMC

Introduction

There are over 800 genes encoding GPCR in the human genome (Fredriksson et al. 2003). Patterns of receptor expression are dependent on cell type, and most cells express many different GPCR (Hakak et al. 2003). Activities of more than 20 GPCR have been described in BSMC, with at least 5 different receptors coupling to $G_{\alpha s}$ (Billington and Penn 2003). Since receptors contribute to compartmentation due to differences in their associations with AC isoforms, we hypothesized that only a subset of receptors in BSMC would be linked to induction of IL-6. Many different receptor types have been implicated in IL-6 induction in various cell types. Among the $G_{\alpha s}$ -coupled receptors that have been linked to IL-6 induction in other cells are β_2 AR, β_3 AR, EP₂R, tachykinin, CGRP, adenosine A2B, and purinergic receptors (Chen et al. 2012; Tchivileva et al. 2009; Fiebich et al. 2000; Millet and Vignery 1997; Wang et al. 2010; Zhong et al. 2005; Inoue et al. 2007). If expressed in the right signaling compartment, these $G_{\alpha s}$ -coupled receptors have the potential to induce IL-6 in BSMC.

One way that AC isoforms differentially regulate cellular responses is through isoform-specific associations with receptors (Ostrom et al. 2000c). Colocalization in specialized membrane microdomains can promote specific AC-receptor interactions. Lipid rafts are specialized domains within the plasma membrane with high levels of cholesterol and sphingolipids. Caveolae represent a subset of lipid rafts distinguishable by their flask-like shape and expression of caveolin proteins. Interactions with membrane lipids or proteins allow enrichment of certain signaling molecules in lipid rafts while others are excluded (Ostrom and Insel 2004). The content of caveolae is dynamic and translocation can alter the regulation and responses of signaling molecules (Ostrom et al. 2001; Rybin et al. 2000). There are likely several mechanisms acting to target specific proteins to lipid rafts and caveolae, including protein-lipid interactions, protein-protein interactions and post-translational modifications (Moffett et al. 2000; Resh 2006; Hnasko and Lisanti 2003). Cell-specific differences in localization of receptors and AC have been observed for both native and overexpressed protein, providing an additional mechanism for cell-specific cAMP-mediated responses (Ostrom et al. 2000c; Ostrom et al. 2002). Colocalization in lipid rafts facilitates specific receptor-AC associations and interactions with downstream effectors and is likely responsible for some receptor-specific and AC isoform-specific cellular responses (Pike 2003).

Particular GPCR-AC associations have been described in a number of different cells, including BSMC. β AR and AC6 are colocalized and interact in caveolae in neonatal cardiac myocytes, and a number of other receptors do not have access to AC6 in this compartment (Ostrom et al. 2000c). In BSMC AC6 and β_2 AR colocalize in caveolae, while AC2 and AC4 are excluded from them. EP₂R colocalize and interact with AC2/AC4 outside of lipid rafts and cannot stimulate AC6 (Bogard et al. 2011). Specific GPCR-AC coupling can also occur through direct protein-protein interactions or AKAP-organized complexes (Lavine et al. 2002; Dessauer 2009). Due to specific

localization and interactions of GPCR with AC and downstream effectors, we hypothesized that not all $G_{\alpha s}$ coupled receptors are associated with AC2 and tied to induction of IL-6 expression. We examined expression of GPCR in BSMC and used specific agonists to activate GPCR to determine which receptors stimulate IL-6 production.

Results

Receptor-mediated induction of IL-6 is enhanced specifically by AC2

After finding that direct activation of AC with Fsk resulted in an induction of IL-6 that was enhanced specifically by AC2 overexpression and not other AC isoforms, we wanted to determine if AC2 specificity persists when IL-6 is induced by activation of $G_{\alpha s}$ -coupled receptors. We hypothesized that colocalization of receptors, AC, and downstream effectors create distinct cAMP signaling compartments in BSMC. We have previously shown that AC2 and EP₂R are excluded from lipid rafts in BSMC, and that β_2 AR are predominately localized to lipid rafts where they colocalize with AC6 (Bogard et al. 2011). These receptors have also been shown to induce IL-6 expression in other cell-types (Chen et al. 2012; Fiebich et al. 2001). Thus, we stimulated β_2 AR with isoproterenol (Iso) or EP₂R with butaprost (Buta) for 24 h and measured IL-6 mRNA by qRT-PCR and IL-6 secreted into culture supernatant by ELISA.

Iso and Buta were both able to induce IL-6 expression in BSMC. In control and AC overexpressing cells these agonists caused a significant increase in IL-6 mRNA (**Figure 4-1**). AC2 overexpression significantly enhanced IL-6 mRNA production in Iso treated cells compared to cells expressing only native levels of AC. Since the effect of Buta on IL-6 induction was highly variable, AC2's effect did not reach statistical significance. AC6 overexpression did not change RNA levels compared to control cells in response to any of the agonists tested.

Treatment with Iso or Buta also increased IL-6 protein in an AC2-specific manner (**Figure 4-2**). Unlike mRNA, these agonists did not significantly increase IL-6 protein compared to vehicle in control cells. Only in AC2 overexpressing cells did Iso and Buta treatments result in significant increases in IL-6 production. AC2 overexpression enhanced IL-6 protein production in response to Iso compared to control cells. As with mRNA studies, variability in the response to Buta prevented a statistically significant enhancement in AC2 overexpressing cells. AC6 overexpression did not alter IL-6 protein levels compared to control cells with endogenous AC expression in any of the conditions tested.

Figure 4-1. IL-6 mRNA expression in response to GPCR agonists

qRT-PCR was used to determine IL-6 mRNA levels in BSMC overexpressing lacZ, AC2 or AC6 treated with 1 μ M of the indicated drug for 1 h. mRNA levels are expressed relative to lacZ basal, mean $\pm$ SEM, n=6. * indicates $p < .05$ compared to basal in lacZ, # indicates $p < .05$ in AC overexpressing cells compared to same treatment in control cells.

Figure 4-2. IL-6 protein production in response to GPCR agonists

ELISA were used to determine IL-6 protein levels in culture medium from BSMC overexpressing lacZ, AC2 or AC6 treated with 1 μ M of the indicated drug for 24 h. Dashed line indicates no change with respect to lacZ basal. IL-6 protein is expressed relative to lacZ basal, mean $\pm$ SEM, n=3. * indicates $p < .05$ compared to lacZ basal, # indicates $p < .05$ in AC overexpressing cells compared to same treatment in control cells.

BSMC express several GPCR

GPCR expression is cell-type dependent and most cells express many different receptors. The full complement of receptors expressed in BSMC has not been described previously. To better understand how AC2 specifically enhances IL-6, we wanted to know whether all $G_{\alpha s}$ -coupled GPCR, or only a subset, are able to stimulate IL-6 gene expression. To determine which GPCR are expressed in BSMC, we used PCR arrays to probe for mRNA of 370 receptors (**Table A-3**). Following amplification, SYBR green melt curves were analyzed, and genes with more than one peak were excluded due to the ambiguity associated with multiple products. It is important to note that the PCR array data are qualitative in this context. Since cDNA for each receptor was amplified with different primers resulting in products of varying lengths, receptors with lower CP values are not necessarily expressed at higher levels. The PCR array served as a starting point to discover all the GPCR expressed by BSMC, but ultimately other approaches must be used to confirm functional receptor expression. mRNA was detected (CP <35) for 227 of the receptors tested, while mRNA was not detected (CP >35) for 81 receptors (**Table A-3**).

The PCR array detected mRNA for β_2 AR, with an average CP of 29.90. Amplification by EP₂R primers was also apparent (CP: 26.59), but since there were multiple peaks on the melt curve the data were not conclusive, and this receptor was not included in **Table A-3**. EP₂R expression in BSMC has been confirmed by other methods. EP₂R immunoblots and the ability of Buta, an EP₂R-selective agonist, to increase cAMP and IL-6 (**Figure 4-1**) indicate that this receptor is expressed in BSMC (Bogard et al. 2011). The CP of other receptors potentially associated with IL-6 induction are listed in **Table 4-1** with their predominant G-protein associations and the agonists used to investigate their activity in BSMC.

Not all receptors are associated with induction of IL-6

We chose a subset of the receptors with readily detectible mRNA and measured cAMP and IL-6 production following stimulation with specific agonists (**Table 4-1**). IL-6 protein in culture medium increased compared to vehicle following 24 h treatment with a purinergic receptor agonist, ATP γ S, or adenosine receptor agonist, 5'-N-ethylcarboxamido-adenosine (NECA, **Figure 4-3A**). Arginine vasopressin (AVP), BRL 37344 (β_3 AR-selective agonist), α -calcitonin gene related peptide (α -CGRP), glucagon, prostaglandin D₂ (PGD₂), and substance P did not significantly increase IL-6 secretion compared to vehicle (**Figure 4-3A**).

We also tested these agonists for their ability to stimulate cAMP production in BSMC. Cells were treated with the same GPCR-selective agonists for 10 min in the presence of a broad-spectrum PDE inhibitor (0.2 mM IBMX). ATP γ S and NECA, agonists that increased IL-6 secretion, did not significantly increase global cAMP levels (**Figure 4-3B**). The cAMP response to NECA may be blunted by antagonism of

Table 4-1. GPCR agonists and their targets in BSMC

Agonist	Receptor	Activity	Affinity	G-protein	CP
ATPγS	P2Y12	Full agonist	5.5 ^a	Gi/Go	40
ATPγS	P2Y11	Full agonist	4.9 – 5.5 ^b	1°: Gq/G11 2°: Gs	29.13
ATPγS	P2Y1	Partial agonist	7.4 ^c	1°: Gq/G11 2°: Gi/Go	34.49
ATPγS	P2Y13	Full Agonist	5.5 ^c	Gi/Go	33.92
AVP	Vasopressin V1B	Full agonist	9.0 – 9.5 ^a	Gq	31.00
AVP	Vasopressin V1A	Full agonist	8.5 – 9.3 ^a	Gq/G11	31.63
AVP	Vasopressin V2	Full agonist	7.9 – 9.1 ^a	Gs	33.55
AVP	OT Receptor	Partial agonist	7.3 – 9.3 ^a	Gi/Go	27.42
BRL 37344	β 3 Adrenergic	Full agonist	6.4 – 7 ^a	1°: Gs 2°: Gi/Go	30.96
αCGRP	AM1	Full agonist	6.0 ^d	1°: Gs 2°: Gi/Go, Gq/G11	RAMP2 : ND CALCR L: 31.76
αCGRP	CGRP	Full agonist	9.7 – 10 ^a	1°: Gs 2°: Gi/Go, Gq/G11	RAMP1 : ND CALCR L: 31.76

Table 4-1. (Continued)

Agonist	Receptor	Activity	Affinity	G-protein	CP
αCGRP	AM2	Full agonist	6.5 – 6.8 ^a	1°: Gs 2°: Gi/Go, Gq/G11	RAMP3 : ND CALCR L: 31.76
αCGRP	AMY1	Full agonist	8.7 – 10.8 ^b	Gs	RAMP1 : ND CALCR : 36.08
αCGRP	AMY3	Full agonist	7.6 – 9.7 ^b	Gs	RAMP3 : ND CALCR : 36.08
αCGRP	AMY2	Full agonist	6.2 – 9.7 ^b	Gs	RAMP: ND CALCR : 36.08
αCGRP	CT	Full agonist	6.2 – 8.4 ^b	1°: Gs 2°: Gq/G11	36.08
Glucagon	GLP-1	Full agonist	6.9 – 7 ^a	Gs	ND
Glucagon	Glucagon	Full agonist	9.0 ^b	Gs	34.68
NECA	A3	Full agonist	7.5 – 8.4 ^a	Gi	38.79
NECA	A2A	Full agonist	6.9 – 8.7 ^a	1°: Gs 2°: Gq/G11	32.47
NECA	A1	Full agonist	5.3 – 8.2 ^a	1°: Gi/Go 2°: Gs Gq/G11	32.01

Table 4-1. (Continued)

Agonist	Receptor	Activity	Affinity	G-protein	CP
NECA	A2B	Full agonist	5.7 – 6.9 ^a	1°: Gs 2°: Gq/G11	27.61
PGD2	EP4	Full agonist	8.7 – 9 ^a	1°: Gs 2°: Gi/Go	27.71
PGD2	DP1	Full agonist	7.8 – 9.2 ^a	Gs	32.92
PGD2	DP2	Full agonist	7.6 – 8.6 ^a	Gi/Go	ND
PGD2	FP	Full agonist	7.7 ^a	1°: Gq/G11 2°: Gs	28.38
PGD2	EP2	Full agonist	5.0 ^a	Gs	26.59
PGD2	EP1	Full agonist	4.7 ^a	1°: Gq/G11 2°: Gi/Go	31.82
Substance P	NK1	Full agonist	8.5 – 10.3 ^a	Gs, Gq/G11	34.49
Substance P	NK2	Full agonist	5.9 – 6.9 ^a	Gs, Gq/G11	32.78
Substance P	NK3	Full agonist	5.0 – 6.0 ^a	Gq/G11	32.75

Notes: Affinities are listed as **a**, pKi; **b**, pEC50; **c**, pIC50; **d**, pKd. Some receptors can couple to multiple G-proteins. 1° represents the primary coupling and 2° represents secondary coupling. ND (not determined) indicates genes that were not tested.

Source: Sharman JL, Benson HE, Pawson AJ, Lukito V, Mpamhanga CP, Bombail V, Davenport AP, Peters JA, Spedding M, Harmar AJ, Nc I (2013) IUPHAR-DB: updated database content and new features. Nucleic Acids Res 41 (Database issue):D1083-1088. doi:10.1093/nar/gks960

Figure 4-3. cAMP and IL-6 protein production in response to G_{α_s} -coupled receptor agonists

BSMC were treated with 1 μ M Fsk, AVP, CGRP, glucagon, PGD₂, substance P; 10 μ M ATP γ S, BRL 37344, NECA. **(A)** ELISA were used to measure IL-6 secreted into culture media from cells treated with the indicated drug for 24 h. **(B)** cAMP was measured by EIA in BSMC that were pretreated with 0.2 mM IBMX prior to 10 min agonist. Dashed line represents no change with respect to vehicle. Data are presented as the fold response over vehicle, mean $\pm$ SEM, n=3-4. * indicates $p < .05$ compared to vehicle.

adenosine receptors by IBMX (Daly et al. 1987). BRL 37344 and glucagon, agonists that did not increase IL-6 production, induced small but non-significant cAMP responses.

IL-6 and cAMP levels are not directly correlated

Since levels of IL-6 production did not appear to directly correlate with global cAMP levels, we further examined the correlation between cAMP and IL-6 using agonists for β_2 AR and EP₂R, receptors whose expression in BSMC and ability to stimulate IL-6 in other cell types have been well described (Bogard et al. 2011; Raychaudhuri et al. 2010; Chen et al. 2012). Simultaneous G_{as} and Fsk stimulation produces a synergistic cAMP response in many cell types that can aid in determining the role of this signaling pathway (Insel and Ostrom 2003; Daly et al. 1982; Darfler et al. 1982; Sutkowski et al. 1994; Ostrom et al. 2000a). AC2 overexpressing cells were treated with 0.1 μ M Iso or Buta alone and with concurrent Fsk (0.1 μ M) treatment to determine if increased cAMP always translates to increased IL-6 production. Lower concentrations of each drug were used in order to observe synergistic effects. Addition of Fsk at the time of Buta stimulation significantly increased cAMP production over agonist alone (**Figure 4-4A**). High variability in Iso-treated cells resulted in a non significant effect of Fsk, but addition of Fsk consistently resulted in at least doubling of cAMP compared to Iso alone. Thus, BSMC express a moderate G_{as} -Fsk synergistic effect. Using these same conditions, we found no increase in IL-6 production (**Figure 4-4B**). Simultaneous G_{as} and Fsk stimulation increases cAMP substantially, but the cAMP increase does not translate to greater IL-6 expression. cAMP induces IL-6 expression in BSMC, but the levels of cAMP and IL-6 are not directly correlated.

Discussion

Agonists that do not have a significant effect on cAMP levels can induce IL-6 (**Figure 4-3**). Even with PDE inhibition, ATP γ S and NECA did not significantly increase cAMP production over basal levels. These two agonists can activate GPCR coupled to G_{aq} pathways in addition to G_{as} . G_{aq} initiates protein kinase C (PKC) and mitogen activated protein kinase (MAPK) signaling pathways, which have been shown to induce IL-6 expression in other cell types. Additionally, PKC regulates AC activity of some isoforms. PKC can activate AC2, even without stimulation by G_{as} or Fsk (Jacobowitz and Iyengar 1994). The ability of ATP γ S and NECA to stimulate IL-6 production without significantly increasing cAMP could be through G_{aq} activated PKC directly activating AC2, or via PKC signaling pathways themselves.

Surprisingly Iso and Buta, agonists targeting receptors that are known to be expressed in BSMC and capable of inducing IL-6 expression, did not significantly increase protein production in control cells. With this known, the other agonists would have been better tested in AC2 overexpressing cells, or had their ability to induce mRNA expression measured instead of protein. We cannot be certain that the agonists that did not show increased protein are not associated with induction of IL-6 gene expression.

Figure 4-4. cAMP and IL-6 production in BSMC by GPCR agonists alone or with concurrent Fsk treatment

(A) Cells were treated with 0.1 μ M of the indicated drug with or without concurrent treatment with 0.1 μ M Fsk for 10 min in the presence of 0.2 mM IBMX. cAMP was measured by EIA. **(B)** Cells were treated with 0.1 μ M of the indicated drug with or without concurrent treatment with 0.1 μ M Fsk. IL-6 in culture media was measured by ELISA following 24 h drug treatment. Dashed line represents no change with respect to basal. Data in both panels are presented as the fold over vehicle, mean $\pm$ SEM, n=3.

The agonists BRL-37344, substance P, and CGRP stimulate IL-6 production in other cells, but they did not increase IL-6 protein production in BSMC (**Figure 4-3**) (Tchivileva et al. 2009; Millet and Vignery 1997; Wang et al. 2010). There are cell-type differences in receptor-mediated induction of IL-6, likely due to differences in receptor expression, signaling intermediates and signaling compartments of cells. $G_{\alpha s}$ -coupled receptors β_2 AR and EP₂R are linked to IL-6 induction in BSMC (**Figure 4-1**). As expected based on Fsk responses AC2 derived-cAMP selectively induces IL-6 in response to GPCR stimulation. Not all $G_{\alpha s}$ receptors are associated with induction of IL-6, likely in part due to localization that places them apart from AC2. In addition to colocalization, we found that differences in regulation, particularly AC2 activation by PKC, could be involved in some of the GPCR-mediated IL-6 induction downstream of AC2.

CHAPTER 5. DOWNSTREAM SIGNALING AND PROMOTER ELEMENTS ASSOCIATED WITH cAMP INDUCTION OF IL-6

Introduction

cAMP induces IL-6 through various signaling pathways, with differences among cell types. The intermediate signaling molecules linking cAMP to IL-6 induction have not been studied in BSMC. In astrocytes, PGE₂ enhances IL-6 expression through G_{as}-coupled EP₄-like receptors and p38 MAPK and PKC pathways (Fiebich et al. 2001). In chondrocytes the same stimulus regulates IL-6 expression through PKA and phosphatidylinositol 3-kinase (PI3K) pathways (Wang et al. 2010). IL-6 induction by β AR activation in neonatal mouse cardiac fibroblasts is Epac-dependent and involves PKC δ and p38 MAPK signaling pathways (Chen et al. 2012). Since it is clear that pathways mediating cAMP induction of IL-6 are cell-type specific, we sought to determine which signaling intermediates are required for Fsk-induced IL-6 expression in BSMC.

cAMP can regulate gene expression through several different signaling pathways and a variety of transcription factors, and by the time the signal has reached the nucleus there are many opportunities for crosstalk among various signaling pathways. The IL-6 promoter contains C/EBP, CRE, NF- κ B, and 2 AP-1 sites (Dendorfer et al. 1994; Akira et al. 1990; Tanabe et al. 1988). Pathways initiated by cAMP have the potential to regulate transcription factor and cofactor binding at each of these sites. The most direct pathway linking cAMP to gene induction is through PKA phosphorylation of CREB bound to the CRE site (Gonzalez and Montminy 1989). CREB is bound to chromatin under unstimulated conditions, but PKA phosphorylation at Ser 133 leads to binding of CREB binding protein (CBP) and recruitment of transcription initiation complexes (Montminy 1997). PKA is not the only kinase that phosphorylates CREB at Ser 133, so other pathways can also regulate transcription initiation at CRE (Shaywitz and Greenberg 1999). AP-1 sites have a consensus sequence that is closely related to the CRE sequence, and CREB can also regulate transcription at these sites (Lee et al. 1987; Lamph et al. 1990). Transcription at AP-1 is also initiated by Fos-Jun dimers, which are activated by MAPK signaling cascades (Karin 1996).

NF κ B is involved in induction of many inflammatory genes. It is sequestered in the cytosol by I κ B and translocates to the nucleus following phosphorylation of I κ B by I κ K kinase (I κ K). I κ K can be activated by any of the pathways that have been implicated in IL-6 induction in other cell types (Hayden and Ghosh 2008). The transcription factor C/EBP is also known as NF-IL-6 (Akira et al. 1990). Most cells express multiple C/EBP isoforms with varying activity. cAMP induces C/EBP translocation to the nucleus (Metz and Ziff 1991). Interestingly, asthmatic ASM do not express C/EBP α , an inhibitory isoform (Roth et al. 2004). The C/EBP site does not appear to be involved in cAMP-mediated IL-6 expression, but it may play a role in increased proliferation of ASM in asthma (Roth et al. 2004). AC isoform-specific localization and interactions are involved in establishing distinct cAMP signaling compartments that regulate precise cellular

responses. We looked downstream of cAMP to uncover the signaling intermediates responsible for transduction of the signal from AC2 to the IL-6 promoter. We used selective cAMP agonists and inhibitors of intermediate kinases and found that PKA and Epac are activated downstream of AC2 and stimulate IL-6 induction via CRE and AP-1 promoter elements.

Results

PKA and Epac signaling induce IL-6 production

To determine what makes AC2-derived cAMP specifically linked to induction of IL-6, we examined the downstream signaling pathways that connect cAMP signals to the nucleus. cAMP transduces signals through activation of PKA and Epac. Using specific cAMP analogs we probed for the role of each of these downstream effector molecules in regulating IL-6 levels. Exposure to 1 μ M 8CPT-2Me-cAMP or 8-Br-cAMP did not significantly increase IL-6 protein secretion over vehicle (**Figure 5-1**). The Epac selective analog, 8CPT-2Me-cAMP, significantly increased IL-6 protein at 10 μ M and 100 μ M, increasing IL-6 by slightly over 50% at both concentrations. At 10 μ M the non-selective analog, 8-Br-cAMP, produced a similar response to 8CPT-2Me-cAMP. However, treatment with 100 μ M 8-Br-cAMP led to a substantially larger response than the Epac selective analog, with a 4-fold increase in IL-6 protein over vehicle. The typical response to 1 μ M Fsk resulted in IL-6 levels greater than any 8CPT-2Me-cAMP concentration and comparable to an 8-Br-cAMP concentration between 10 μ M and 100- μ M.

To determine if PKA activity is required for induction of IL-6 in response to Fsk, we pretreated cells with the PKA inhibitor H-89. PKA inhibition reduced IL-6 produced in response to 1 μ M Fsk by 13.4% (**Figure 5-2**). When treated with selective cAMP analogs, PKA had a greater role in regulating induction of IL-6 when cAMP levels were high. Increasing the Fsk concentration to 10 μ M did not result in any greater reduction of IL-6 production by H-89 (**Figure 5-2**). 10 μ M Fsk produces maximal cAMP production in BSMC, but the intracellular cAMP levels are probably not as high as treatment with 100 μ M 8-Br-cAMP. Epac and PKA are responsible for cAMP-mediated induction of IL-6. PKA produces large increases in IL-6 in response to high cAMP levels, but Epac appears to regulate much of the IL-6 expression induced by Fsk treatment.

Inhibition of PKC, p38 MAPK, or PI3K does not block Fsk induction of IL-6

We used inhibitors of PKC (GF 109203X), p38 MAP kinase (SB 202190), or PI3K (wortmanin) to probe for the role of these signaling pathways, since work in other cell types implicates them in $G_{\alpha s}$ -mediated induction of IL-6 (Fiebich et al. 2001; Wang et al. 2010). BSMC were pretreated for 1 h with 10 μ M of each inhibitor, concentrations that significantly inhibited IL-6 expression in other cell types (Fiebich et al. 2001;

Figure 5-1. IL-6 protein production induced by cAMP analogs

ELISA were used to measure IL-6 protein in the culture medium of BSMC following 24 h treatment with the indicated concentration of 8-Br-cAMP or 8CPT-2Me-cAMP. Dashed line represents no change with respect to vehicle. Data are presented relative to vehicle, mean $\pm$ SEM, n=4. * indicates $p < .05$ compared to vehicle, # indicates $p < .05$ compared to 8CPT-2Me-cAMP.

Figure 5-2. IL-6 protein production by Fsk in the presence of PKA inhibitor
ELISA were used to measure IL-6 in culture supernatant of cells pretreated with 3 μM H-89 or vehicle prior to 24 h 1 μM Fsk. Dashed line represents no change with respect to vehicle. Data are presented relative to vehicle, Mean ± SEM, n=4. * indicates p<.05 compared to Fsk alone.

Wang et al. 2010). Following inhibitor pretreatment, cells were exposed to 1 μ M Fsk for 1 h, and IL-6 mRNA expression was measured by qRT-PCR. None of the inhibitors tested had a significant inhibitory effect on Fsk-stimulated IL-6 expression (**Figure 5-3**). In fact, GF 109203X or wortmanin pretreatment tended to increase IL-6 production compared to Fsk alone (not significant). Since nonspecific effects could occur at the concentrations we chose, we tested each inhibitor at concentrations ranging from 10 nM to 10 μ M. There was no inhibition of Fsk-induced IL-6 expression by this range of concentrations by any of the inhibitors tested (data not shown). If anything, these inhibitors slightly increased Fsk-induced IL-6 expression. cAMP-mediated regulation of IL-6 expression may involve multiple stimulatory and inhibitory pathways with substantial cross talk and redundancy, making it difficult to unravel the steps with inhibition of individual intermediate kinases.

Activation of the IL-6 promoter by Fsk requires AP-1 and CRE sites

We used promoter analysis as an alternative approach to determine the signaling pathways involved in cAMP-mediated IL-6 expression. BSMC were transfected with vectors containing 1168 base pairs of the wild type human IL-6 promoter driving expression of the luciferase reporter gene. We also utilized individual promoter-reporter constructs that contained mutations in each of the defined promoter elements in order to test which transcriptional site(s) are required for cAMP-mediated expression of IL-6. The IL-6 promoter contains 2 AP-1 sites and 1 CRE, C/EBP and NF κ B site. Site directed mutagenesis prevented transcription factor binding at the indicated site. Mutagenesis of the 3' AP-1 site increased IL-6 production in vehicle- and Fsk-treated BSMC, possibly due to regulation by a repressor at this site in the wild type promoter (**Figure 5-4**). Promoter activity was unchanged in the other mutant constructs under basal conditions. When stimulated with 1 μ M Fsk, luciferase activity was significantly reduced when the 5' AP-1 or CRE site was mutated as compared to the wild type promoter (**Figure 5-4**). Mutations to C/EBP or NF- κ B did not alter the promoter's response to Fsk. For cAMP induction of IL-6 in BSMC, 5' AP-1 and CRE sites are essential but C/EBP and NF κ B sites are not involved.

Discussion

At lower cAMP concentrations, Epac-selective and non-selective cAMP analogs have similar effects on IL-6 protein production (**Figure 5-1**). At higher cAMP concentrations, Epac does not contribute to any additional IL-6 response, but PKA signaling has a large effect on inducing IL-6 production. These data indicate a high affinity but low efficacy Epac component and a lower affinity but higher efficacy contribution from PKA activity. Stimulation by 1 μ M Fsk likely produces intermediate cAMP levels that act through both Epac and PKA to induce IL-6 expression. PKA inhibition with H-89 resulted in a small reduction of IL-6 protein in response to 1 μ M or 10 μ M Fsk (**Figure 5-2**), consistent with dual pathways employing Epac or PKA.

Figure 5-3. Fsk-induced IL-6 expression in the presence of kinase inhibitors
qRT-PCR was used to measure IL-6 mRNA in BSMC pretreated with 10 μ M of the indicated inhibitor prior to 1 μ M, 1 h Fsk exposure. Data are expressed relative to vehicle, mean $\pm$ SEM, n=3-4.

Figure 5-4. Promoter activity of wild type and mutant IL-6 promoter constructs
 Luciferase assays were used to measure promoter activity in BSMC transfected with wild type or mutant promoter constructs and exposed to 1 μ M Fsk or vehicle for 6 h. Wild type is 1168 base pairs of the human pIL-6 promoter. Each promoter mutant construct is named by the binding site that has been ablated by site directed mutagenesis. Data are expressed as luminescence relative to wild type basal, mean $\pm$ SEM, n=11. * indicates p<.05 compared to wild type.

The kinase inhibitors that we used to try to understand the signaling intermediates involved did not reduce Fsk-induced IL-6 expression in BSMC (**Figure 5-3**). It is possible that some other pathway is responsible for activating IL-6 expression, or the inhibitors that were effective in other cell types may not be appropriate for inhibiting these pathways in BSMC. By the time the signal has been passed from the plasma membrane to the nucleus there are many chances for cross-talk and redundancy of pathways, and inhibition at a single point may not be enough to prevent induction. Another pitfall is that inhibitors are not highly selective. An alternative approach to pharmacological inhibitors is RNA interference, which could produce specific inhibition without off-target effects. The poor transfection efficiency of BSMC limits the utility of RNAi in the present studies. We found that PKA and Epac are involved in cAMP-mediated induction of IL-6 in BSMC, but have little insight into the downstream effectors that are involved in the signal transduction pathways leading to activation of transcription at CRE and AP-1 promoter sites.

CHAPTER 6. FINAL DISCUSSION AND FUTURE DIRECTIONS

AC Isoform-Specific Responses

This work represents the first demonstration of AC isoform-specific control of gene regulation. Previous studies have defined cAMP signaling compartments in near-membrane events or regulation of proximal intracellular signals, but no other work of which we are aware has demonstrated cAMP compartments regulating specific genes. AC2 contributes to a signaling compartment that selectively induces IL-6 in BSMC. AC6-derived cAMP is involved in regulation of other genes, including SST, which it selectively regulates, but it does not influence IL-6 expression. Differences in AC regulation, localization, and associations with other proteins allow AC isoforms to create unique cAMP pools with diffusion constrained by activity of PDE. Through activation of Epac and PKA, cAMP produced by AC2 signals to the nucleus and induces IL-6 expression via CRE and AP-1 promoter elements.

Inherent differences in regulation of AC2 and AC6 could be one contributor to AC2 selective IL-6 enhancement. AC2 is activated by PKC, but AC6 is inhibited by this kinase (Jacobowitz and Iyengar 1994; Lai et al. 1999). The ability of ATP γ S and NECA to induce IL-6 without significant global cAMP increase (**Figure 4-3**) could be explained by activation of G $_{\alpha q}$ -coupled receptors, which stimulate a direct PKC activation of AC2. These molecules may all exist as part of a complex with AKAP79 as recently described by Shen and Cooper (2013). Using plasma membrane targeted Epac2 FRET-based cAMP sensors along with molecular and pharmacological approaches, they showed that activation of muscarinic receptors leads to the receptor's association with AKAP79 in HEK-293 cells. AKAP79 forms a complex containing the receptor, AC2, PKC, PKA, and PDE4. This complex results in cAMP signals that are confined in space and time. Upon stimulation of the muscarinic receptor, PKC activates AC2 (a unique feature of this class of AC isoforms). The cAMP produced by AC2 is short-lived since it activates PDE4 activity via PKA, and all components are kept in close association by AKAP79 (Shen and Cooper 2013). With such tightly controlled cAMP production and degradation, downstream effectors must be in close proximity to the AC in this case.

It is likely that AC6 is also part of signaling complexes containing PDE. PDE4 appears to play a role in preventing cAMP produced by AC6 from inducing IL-6 expression. When we treated BSMC with the PDE4-selective inhibitor rolipram, overexpression of AC6 significantly increased IL-6 production, even at basal AC activity (with no addition of Fsk, data not shown). The AKAP gravin orchestrates a complex with PDE4 to restrict cAMP to the membrane region (Willoughby et al. 2006). Given that gravin is expressed in airway smooth muscle one might hypothesize that this complex acts to restrict the AC6 signaling compartment (Horvat et al. 2012). Further work should seek to define all the proteins associated with the AC isoforms natively expressed in BSMC and uncover cellular responses mediated by these different complexes.

Downstream Signaling Pathways

Our data indicate that AC2-derived cAMP activates PKA and Epac (**Figure 5-1**), which are both involved in regulating IL-6 expression. At low levels of cAMP, Epac is important for transducing the signal, and at higher cAMP levels PKA plays a predominant role. These responses may reflect different sensitivities of PKA and Epac for cAMP or may also be reflective of how these two effectors participate in this specific cAMP compartment. Further studies are needed to fully understand the different roles of PKA and Epac.

Measures of bulk intracellular cAMP levels are not correlated with the amount of IL-6 produced (**Figures 4-3 and 4-4**). Different receptors that stimulated both cAMP production and IL-6 did not show a clear relationship between cAMP levels and IL-6. In some cases, this could be explained by activation of other GPCR that stimulate $G_{\alpha q}$ and PKC. However, $G_{\alpha s}$ -Fsk synergism using only $G_{\alpha s}$ -coupled receptors led to increased cAMP production with no further increase in IL-6 production. It is possible that there is a threshold after which additional cAMP has no ability to further increase IL-6 expression. Alternatively, a cAMP compartment model could also explain the lack of correlation with large amounts of cAMP. If cAMP is not produced in the right location, it may be unable to activate the pathways that enhance IL-6 expression. Further studies that can detect cAMP produced in distinct subcellular compartments are needed to understand if such pools of cAMP exist.

We sought to determine which downstream kinases are involved in induction of IL-6 expression with the intent of determining if they are differentially activated when AC2 or AC6 is overexpressed and activated. From the work of others it is apparent that cAMP-mediated induction of IL-6 occurs through different pathways based on the cell-type and stimulus. We used pharmacological inhibitors of three kinases that are associated with cAMP-mediated induction of IL-6 in different cell-types. GF109203X (PKC inhibitor), SB202190 (p38 MAPK inhibitor), and wortmanin (PI3K inhibitor) did not inhibit IL-6 expression in response to Fsk, even when a range of concentrations was tested (**Figure 5-3**). These kinases that were required for IL-6 induction in other cells are not essential for Fsk-induced IL-6 production in BSMC. In fact, our data imply that these pathways may tonically inhibit the induction of IL-6. There also may be significant crosstalk among signaling pathways, so inhibition of a single intermediate does not prevent signaling to the nucleus and induction of IL-6 expression. PKC, PI3K or p38 could be part of the signaling cascade that links surface receptors to CRE and AP-1 sites of the IL-6 promoter, but other pathways predominate when one component is inhibited. A schematic of the possible signal transduction pathways connecting activation of AC2 to induction of IL-6 expression is shown in **Figure 6-1**.

RNA Stabilization

Throughout this work, we focused on AC2-specific enhancement of *induction* of IL-6 transcription. The ability of cAMP to regulate gene expression through CRE and

Figure 6-1. cAMP-mediated induction of IL-6 in BSMC

Green circles represent cAMP and purple ovals represent downstream effectors. Following stimulation by Fsk or GPCR agonists, AC2-derived cAMP activates Epac and PKA in a signaling compartment that allows transduction of the signal to the nucleus and induction of transcription of IL-6. PDE prevent AC6-derived cAMP from activating the necessary intermediates for inducing IL-6 expression. cAMP stimulates IL-6 expression through AP-1 and CRE sites. PKA-phosphorylated CREB can activate transcription AP-1 and CRE sites, and CREB can also be phosphorylated by kinases PKC, calmodulin kinase, and RSK2 (Wen et al. 2010). Transcription at AP-1 sites can be stimulated by Fos/Jun through MAPK signaling pathways. There are likely many signaling pathways driving IL-6 expression in response to cAMP.

AP-1 promoter elements is well established (Montminy 1997). However, careful examination of all the data from promoter activity, mRNA levels, and protein production it becomes apparent that AC2 might also act to stabilize IL-6 mRNA and not just impact promoter activation and transcription of IL-6. In control cells Fsk induces expression of IL-6 mRNA. It increases promoter activity by approximately 3-fold, mRNA by 6.5-fold at 1h, and protein by 2-fold at 24 h (**Figures 5-4 and 3-6**). When AC2 is overexpressed, IL-6 mRNA is increased 77-fold over cells with endogenous AC expression following exposure to 1- μ M Fsk for 24 h (**Figure 3-1**). At 1 h, the effect of AC2 overexpression on Fsk-induced IL-6 mRNA levels is less robust, with only a doubling of IL-6 mRNA compared to control (**Figure 3-7**). The effect of AC2 overexpression on IL-6 abundance is largest at the level of mRNA and has a smaller impact on promoter activation and protein production, at least with the methods we have used. cAMP certainly does induce transcription of IL-6 in BSMC, but the enhanced response with overexpression and activation of AC2 may also be due to activity that stabilizes IL-6 RNA.

For some genes cAMP has been shown to increase mRNA levels via two distinct mechanisms: increasing the rate of transcription and acting to reduce degradation of transcripts (Hod and Hanson 1988). cAMP-mediated stabilization of lactate dehydrogenase (LDH) A subunit mRNA involves binding of a complex of PKA-phosphorylated proteins to the cAMP-stabilizing region (CSR) in the 3'-untranslated region. The formation of the RNA stabilizing complex requires AKAP95, so AC isoform-specific interactions are likely involved in this process too (Jungmann and Kiryukhina 2005). Future studies should determine the stability of IL-6 transcripts in control and AC overexpressing BSMC to see if AC2-derived cAMP specifically stabilizes IL-6 mRNA. Addition of a transcription inhibitor such as Actinomycin D following Fsk exposure would allow comparisons of IL-6 stability in control and AC overexpressing cells.

Changes in Disease

BSMC from an asthmatic patient had basal levels of IL-6 mRNA that were 3-times higher than BSMC from a non-asthmatic donor (**Figure 3-11**). When BSMC from the asthmatic donor were treated with 1 μ M Fsk, there was no increase in IL-6 mRNA compared to those cells untreated. In non-asthmatic BSMC treated at the same time, Fsk caused a 2.6-fold increase in IL-6 mRNA levels. There could be changes in the cAMP signaling compartments or in downstream effector pathways that result from the disease. BSMC from asthmatics are known to have changes in the expression of at least one C/EBP isoform, which results in higher IL-6 expression in response to rhinovirus infection (Oliver et al. 2006).

While we observed differences in untreated and Fsk-induced IL-6 expression by BSMC from normal versus asthmatic donors, this study must be repeated with additional donors to confirm the differences are due to asthma. Future work should examine whether there are differences in relative expression and activity of different AC isoforms among individuals and in disease. Studies to characterize the AC isoforms expressed,

their localization, and relative activities should be repeated in diseased BSMC to determine if there are any intrinsic differences in the cAMP signaling compartments of asthmatic BSMC.

Phosphoproteomics

The project described here has provided as many questions as answers. We are the first to show AC isoform-specific differences in regulation of gene expression, but have little insight into the mechanistic details behind this phenomenon. By the time a cAMP-mediated signal has reached the nucleus there have been many opportunities for stimulatory and inhibitory signaling and cross talk among signaling pathways, which make it difficult to trace an AC2-derived cAMP signal all the way to the IL-6 promoter. Future work will look at differences downstream of individual AC isoforms that are more proximal to cAMP production.

We are currently awaiting results from a phosphoproteomics study to identify differences in phosphorylation patterns when different AC isoforms are overexpressed and activated in BSMC. We used stable isotope labeling by amino acids in cell culture (SILAC) to prepare samples for quantitative phosphoproteomic analysis. **Figure 6-2** illustrates a sample SILAC workflow. SILAC involves culturing two groups of cells in parallel in custom media containing isotope labeled arginine and lysine. The first group is grown in medium with unlabeled, “light” amino acids. The second group is grown in medium with arginine and lysine containing exclusively “heavy” non-radioactive ^{13}C and ^{15}N . Cells are grown in the “light” or “heavy” medium for at least 5 passages, which results in complete incorporation of these amino acids and all proteins in the cell are isotopically labeled (Ong and Mann 2006). For our study lacZ, AC2, or AC6 overexpressing BSMC grown in “light” medium were treated with 1 μM Fsk for 10 min to stimulate protein phosphorylation. The corresponding cells in “heavy” medium were vehicle-treated. Following treatment, cells were lysed in a buffer containing protease and phosphatase inhibitors. Total protein in sonicated lysate was determined by BCA protein assay, and equal amounts of protein from vehicle- and Fsk- treated samples from cells overexpressing the same AC were combined. Samples were sent to the Mass Spectrometry and Proteomics Facility at Johns Hopkins for phosphoprotein enrichment and mass spectrometry analysis. Analysis of these samples will identify the phosphoproteins increased in response to Fsk treatment, and AC overexpressing cells will be compared to control BSMC. Isotope labeling allows direct comparison between vehicle and Fsk-treated cells, since the two samples are combined equally and prepared and analyzed simultaneously. Proteins in lysate of Fsk-treated cells are “light” at every lysine and arginine, so the mass to charge ratio of all trypsin-digested peptides from Fsk-treated cells will be shifted to the left compared to “heavy” peptides from vehicle-treated cells (see **Figure 6-2**). Relative intensities of each peptide can be compared to determine whether cells with native AC expression and overexpressing AC2 or AC6 display increased phosphorylation of that protein in response to 1 μM Fsk. The relative ratios of intensity for different proteins can be compared in lacZ, AC2, and AC6 overexpressing cells to determine if phosphorylation patterns differ with overexpression and activation of

Figure 6-2. SILAC experimental setup

(a) Adaptation phase: “heavy” amino acids are incorporated over time with 100% incorporation after five doublings. **(b) Experimental phase:** Following treatment cells are lysed and combined 1:1 based on total protein. Samples are fractionated and analyzed by mass spectrometry. m/z of peptides from cells grown in “heavy” medium are shifted to the right. Relative intensity of “heavy” to “light” peak gives relative abundance of peptide in treated compared to control samples.

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a Adaptation phase

b Experiment phase

individual AC isoforms. Results from the SILAC phosphoproteomics study may provide insight into signaling intermediates activated by AC2 that could induce IL-6. Results will also be a starting point for uncovering additional AC isoform-specific cellular responses in BSMC.

AC Isoform-Specific Regulation of Other Genes

This project primarily focused on regulation of IL-6, but the PCR array data include other genes that are differentially regulated by individual AC isoforms that could be of interest in regard to cAMP signaling compartments and human health (**Table A-1**). With more information on the phosphorylation patterns downstream of individual AC from our phosphoproteomics study, pathways linking specific AC isoforms to gene regulation may become apparent. Another gene that appears to be specifically regulated by AC2-derived cAMP is prostaglandin-endoperoxide synthase 2, also known as cyclooxygenase 2 (COX-2). COX enzymes catalyze the first step in conversion of arachidonic acid to prostanoids, and are important in the pathogenesis of several inflammatory diseases (Smith et al. 2000). COX-1 is constitutively expressed, but COX-2 is inducible, and its expression is upregulated in inflammatory diseases and many types of cancer (Vane et al. 1998). IL-1 β is one of the proinflammatory cytokines present at higher levels in asthmatic airway, and it is one inducer of COX-2 expression in BSMC (Pang and Knox 1997). COX-2 can also be induced by PGE₂, one product of COX-mediated arachidonic acid metabolism, via cAMP signaling pathways (Faour et al. 2008). The pathologic consequences of COX-2 induction can be appreciated by observing the beneficial anti-inflammatory effects of COX-2 selective inhibitors such as Celecoxib (Celebrex) for treatment of chronic inflammation in patients with osteoarthritis and rheumatoid arthritis (Vane et al. 1998; FitzGerald and Patrono 2001).

Work from our lab has shown that cAMP-mediated cellular responses can be regulated by specific AC isoforms. This project has demonstrated that individual AC isoforms regulate expression of some cAMP-responsive genes. One gene that is of particular interest in the pathogenesis of asthma is the pro-inflammatory cytokine IL-6, which is induced by AC2- but not AC6-derived cAMP. β AR agonists that are used to treat asthma may induce IL-6 via AC2 activity. As more is learned about compartmentation of cAMP signaling and attributes of AC isoform-specific pharmacological tools, individual AC isoforms have the potential to become therapeutic targets. Our SILAC study could add greatly to the field as it may identify specific patterns of kinase activity downstream of individual AC isoforms in BSMC. Pharmaceuticals target GPCR for treatment of many diseases through activation of cAMP signaling pathways. Receptors can be promiscuous in their coupling, allowing activation of various unintended pathways that can worsen conditions the agonists are meant to treat. Targeting AC directly would allow activation of cAMP signaling pathways without the chance of G_{ai} or G_{aq} activation. Work such as ours may allow even more precise targeting of therapeutics by identifying the individual AC isoforms and cAMP signaling compartments that contribute to beneficial responses for treatment of disease without activation of undesirable pathways and adverse effects.

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APPENDIX. PCR ARRAYS

Table A-1. Fsk-induced gene regulation in AC overexpressing BSMC compared to control BSMC

Gene	Description	AC2		AC3		AC6	
		Fold Change	SEM	Fold Change	SEM	Fold Change	SEM
ADRB1	Adrenergic, beta-1-, receptor	-0.445	0.956	-0.302	0.872	1.496	0.152
AHR	Aryl hydrocarbon receptor	1.458	1.595	-1.276	1.189	-1.957	1.673
AMD1	Adenosylmethionine decarboxylase 1	1.040	1.421	-2.709	2.176	-2.241	2.075
AREG	Amphiregulin	3.743	2.157	1.099	2.588	10.334	4.347
ATF3	Activating transcription factor 3	2.749	1.071	-1.074	1.261	-2.488	0.733
BCL2	B-cell CLL/lymphoma 2	0.534	1.124	-1.454	0.368	0.698	1.064
BDNF	Brain-derived neurotrophic factor	0.864	1.048	-1.130	1.347	-0.367	1.123
BRCA1	Breast cancer 1, early onset	0.849	1.316	-0.459	0.808	-2.474	0.124
CALB1	Calbindin 1, 28kDa	-0.445	0.956	-0.315	0.880	1.366	0.213
CALB2	Calbindin 2	-0.876	1.215	0.393	0.708	0.559	0.925
CALM1	Calmodulin 1 (phosphorylase kinase, delta)	1.772	0.194	-0.774	1.043	-1.859	1.759
CALR	Calreticulin	0.634	1.049	-0.240	1.354	-6.404	6.629
CCNA1	Cyclin A1	1.806	1.753	0.855	1.446	-1.200	1.267

Table A-1. (Continued)

Gene	Description	AC2		AC3		AC6	
		Fold Change	SEM	Fold Change	SEM	Fold Change	SEM
CCND1	Cyclin D1	0.608	1.234	-1.668	1.467	-3.416	1.452
CDK5	Cyclin-dependent kinase 5	0.580	1.385	-1.828	1.691	-1.970	0.534
CDKN2B	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	2.161	0.534	-1.101	1.354	-2.088	0.364
CGA	Glycoprotein hormones, alpha polypeptide	0.412	0.886	0.415	0.776	0.472	0.964
CHGA	Chromogranin A (parathyroid secretory protein 1)	-0.450	0.958	-0.315	0.880	1.480	0.155
CNN1	Calponin 1, basic, smooth muscle	-1.138	1.204	-1.691	0.144	-1.391	0.173
CREB1	CAMP responsive element binding protein 1	0.449	1.351	-0.946	1.042	-0.813	0.927
CREM	CAMP responsive element modulator	0.864	1.433	-0.939	1.242	1.562	0.104
CTF1	Cardiotrophin 1	0.297	0.860	-0.664	0.973	-2.368	0.784
CYR61	Cysteine-rich, angiogenic inducer, 61	1.121	1.233	0.378	0.892	-2.096	0.346
DDIT3	DNA-damage-inducible transcript 3	1.856	0.485	0.468	0.875	0.785	1.794
DUSP1	Dual specificity phosphatase 1	3.385	2.744	-1.144	1.823	-0.839	1.644
EGR1	Early growth response 1	3.694	2.382	-0.188	1.958	-2.223	1.977

Table A-1. (Continued)

Gene	Description	AC2		AC3		AC6	
		Fold Change	SEM	Fold Change	SEM	Fold Change	SEM
EGR2	Early growth response 2	1.223	1.232	-0.315	0.880	1.527	0.151
ENO2	Enolase 2 (gamma, neuronal)	0.791	1.138	-2.244	1.953	-1.607	0.201
FGF6	Fibroblast growth factor 6	-0.445	0.956	-0.315	0.880	1.366	0.213
FOS	FBJ murine osteosarcoma viral oncogene homolog	3.736	1.400	-0.666	2.957	3.687	2.110
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	0.430	1.613	-0.602	0.854	-0.433	0.847
GCG	Glucagon	-0.445	0.956	-0.315	0.880	1.366	0.213
GEM	GTP binding protein overexpressed in skeletal muscle	0.406	1.102	-1.401	1.323	-0.361	0.994
GIPR	Gastric inhibitory polypeptide receptor	-2.301	0.570	-0.739	0.936	-0.791	0.956
HK2	Hexokinase 2	0.168	0.985	-1.086	1.211	-0.717	0.883
HSPA4	Heat shock 70kDa protein 4	-0.491	0.811	-1.689	0.571	-2.123	0.620
HSPA5	Heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	2.143	2.234	-1.599	1.515	-1.335	1.195
IL2	Interleukin 2	-0.445	0.956	-0.315	0.880	1.366	0.213
IL6	Interleukin 6 (interferon, beta 2)	77.159	50.029	0.115	1.667	-0.469	0.747
INHBA	Inhibin, beta A	2.066	1.850	-1.135	1.072	0.462	0.758

Table A-1. (Continued)

Gene	Description	AC2		AC3		AC6	
		Fold Change	SEM	Fold Change	SEM	Fold Change	SEM
JUNB	Jun B proto-oncogene	0.721	1.078	-1.579	1.499	-1.956	0.516
JUND	Jun D proto-oncogene	0.171	1.050	-2.122	0.706	-0.787	1.003
KCNA5	Potassium voltage-gated channel, shaker-related subfamily, member 5	-0.445	0.956	-0.315	0.880	1.366	0.213
LDHA	Lactate dehydrogenase A	-0.486	1.901	-1.323	1.239	0.300	0.815
MAF	V-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)	-2.022	0.453	-1.092	1.114	-0.290	0.865
MIF	Macrophage migration inhibitory factor (glycosylation-inhibiting factor)	0.268	1.040	-0.595	0.912	1.283	0.139
NCAM1	Neural cell adhesion molecule 1	-0.445	0.956	-0.315	0.880	1.366	0.213
NF1	Neurofibromin 1	-1.347	0.156	0.187	0.991	-1.265	0.163
NOS2	Nitric oxide synthase 2, inducible	-0.445	0.956	-0.315	0.880	1.366	0.213
NPY	Neuropeptide Y	0.499	0.875	-0.501	0.798	1.364	0.078
NR4A2	Nuclear receptor subfamily 4, group A, member 2	6.710	4.372	-0.419	1.573	2.391	0.833
PCK2	Phosphoenolpyruvate carboxykinase 2 (mitochondrial)	-0.857	1.051	-2.836	0.818	-0.605	1.203

Table A-1. (Continued)

Gene	Description	AC2		AC3		AC6	
		Fold Change	SEM	Fold Change	SEM	Fold Change	SEM
PCNA	Proliferating cell nuclear antigen	0.728	1.291	-1.772	0.314	-2.075	0.511
PENK	Proenkephalin	0.621	1.048	-1.324	0.099	-1.358	0.156
PER1	Period homolog 1 (Drosophila)	-1.256	0.156	-2.071	0.385	-0.439	0.738
PLAT	Plasminogen activator, tissue	0.541	1.753	-1.854	0.592	-1.013	1.131
PLN	Phospholamban	-1.680	0.379	-1.536	0.169	0.864	1.161
PMAIP1	Phorbol-12-myristate-13-acetate-induced protein 1	0.617	1.374	-1.889	0.682	1.621	0.026
POU1F1	POU class 1 homeobox 1	-0.445	0.956	0.384	0.873	1.643	0.317
POU2AF1	POU class 2 associating factor 1	-0.398	0.939	-0.377	0.770	1.252	0.107
PPP1R15A	Protein phosphatase 1, regulatory (inhibitor) subunit 15A	1.046	1.763	-1.422	1.380	-2.866	2.936
PPP2CA	Protein phosphatase 2, catalytic subunit, alpha isozyme	0.643	0.896	-2.441	0.794	-1.167	1.202
PRKAR1A	Protein kinase, cAMP-dependent, regulatory, type I, alpha (tissue specific extinguisher 1)	-0.265	1.289	-2.188	0.867	-1.532	0.220
PRL	Prolactin	-0.445	0.956	-0.315	0.880	1.757	0.394

Table A-1. (Continued)

Gene	Description	AC2		AC3		AC6	
		Fold Change	SEM	Fold Change	SEM	Fold Change	SEM
PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	11.294	7.522	-0.737	1.199	2.560	1.004
RB1	Retinoblastoma 1	1.273	0.096	-0.440	0.973	-0.738	0.965
S100A12	S100 calcium binding protein A12	-0.445	0.956	-0.315	0.880	1.366	0.213
S100A6	S100 calcium binding protein A6	-1.747	0.510	-1.924	0.186	-1.225	0.100
S100G	S100 calcium binding protein G	2.214	0.627	-0.157	0.932	2.320	0.790
SCG2	Secretogranin II	-4.204	3.440	-0.944	1.056	2.598	0.189
SGK1	Serum/glucocorticoid regulated kinase 1	0.397	0.894	-0.743	0.956	1.807	1.732
SLC18A1	Solute carrier family 18 (vesicular monoamine), member 1	-0.445	0.956	-0.315	0.880	1.366	0.213
SOD2	Superoxide dismutase 2, mitochondrial	-1356.5	1369.7	-0.267	0.969	-2.159	2.664
SRF	Serum response factor (c-fos serum response element-binding transcription factor)	0.458	0.864	-0.997	1.083	-0.522	1.215

Table A-1. (Continued)

Gene	Description	AC2		AC3		AC6	
		Fold Change	SEM	Fold Change	SEM	Fold Change	SEM
SST	Somatostatin	-0.826	1.466	1.072	1.283	9.737	0.791
SSTR2	Somatostatin receptor 2	0.363	0.989	-1.342	0.025	2.003	0.487
STAT3	Signal transducer and activator of transcription 3 (acute-phase response factor)	0.434	0.900	-1.369	0.011	-0.535	0.982
TACR1	Tachykinin receptor 1	0.441	1.027	-1.600	0.304	0.820	1.353
TGFB3	Transforming growth factor, beta 3	-0.301	0.831	-1.523	0.295	0.981	1.059
TH	Tyrosine hydroxylase	-0.445	0.956	-0.315	0.880	1.366	0.213
THBS1	Thrombospondin 1	-1.681	0.140	-1.649	0.055	-0.792	0.956
TNF	Tumor necrosis factor	-0.445	0.956	-0.315	0.880	1.366	0.213
VCL	Vinculin	-0.340	1.321	-0.570	1.212	0.836	1.330
VIP	Vasoactive intestinal peptide	1.910	0.520	-1.575	0.514	0.169	1.120

Notes: BSMC were treated with 1 μ M Fsk for 24 h. Gene expression is expressed as fold change with respect to lacZ.

Table A-2. Fsk-induced gene regulation in AC overexpressing HEK compared to control HEK

Gene	Description	AC2	AC6
ADRB1	Adrenergic, beta-1-, receptor	1.879	1.3472
AHR	Aryl hydrocarbon receptor	1.2142	1.4241
AMD1	Adenosylmethionine decarboxylase 1	1.257	-1.0425
AREG	Amphiregulin	1.014	1.4948
ATF3	Activating transcription factor 3	1.1892	1.366
BCL2	B-cell CLL/lymphoma 2	1.0718	1.1173
BDNF	Brain-derived neurotrophic factor	1.4743	1.0644
BRCA1	Breast cancer 1, early onset	1.0792	1.2058
CALB1	Calbindin 1, 28kDa	1.5583	-1.057
CALB2	Calbindin 2	1.5583	-1.057
CALM1	Calmodulin 1 (phosphorylase kinase, delta)	1.5583	1.1251
CALR	Calreticulin	1.9453	1.1173
CCNA1	Cyclin A1	1.3947	1.6586
CCND1	Cyclin D1	-148.0561	-1.4142
CDK5	Cyclin-dependent kinase 5	1.2226	1.1019
CDKN2B	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	1.4044	1.0497
CGA	Glycoprotein hormones, alpha polypeptide	1.1728	1.7654
CHGA	Chromogranin A (parathyroid secretory protein 1)	1.0497	-1.0943
CNN1	Calponin 1, basic, smooth muscle	-1.0718	-3.0951
CREB1	CAMP responsive element binding protein 1	1.3195	1.2226
CREM	CAMP responsive element modulator	1.2226	1.4845
CTF1	Cardiotrophin 1	1.9453	-1.4743
CYR61	Cysteine-rich, angiogenic inducer, 61	2.1435	-1.0644
DDIT3	DNA-damage-inducible transcript 3	1.7654	1.879
DUSP1	Dual specificity phosphatase 1	1.0497	1.0718
EGR1	Early growth response 1	1.0644	1.0943
EGR2	Early growth response 2	2.4794	-1.1408
ENO2	Enolase 2 (gamma, neuronal)	-1.0497	-1.6358
FGF6	Fibroblast growth factor 6	1.7171	-1.057
FOS	FBJ murine osteosarcoma viral oncogene homolog	-1.014	-1.0353

Table A-2. (Continued)

Gene	Description	AC2	AC6
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	1.1487	1.2924
GCG	Glucagon	1.5583	-1.057
GEM	GTP binding protein overexpressed in skeletal muscle	1.8532	1.6472
GIPR	Gastric inhibitory polypeptide receptor	3.9177	1.3287
HK2	Hexokinase 2	2	-1.0644
HSPA4	Heat shock 70kDa protein 4	1.7777	-1
HSPA5	Heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	-1.0644	1.6133
IL2	Interleukin 2	1.5583	-1.057
IL6	Interleukin 6 (interferon, beta 2)	1.5583	1.6586
INHBA	Inhibin, beta A	-1.0718	1.9053
JUNB	Jun B proto-oncogene	1.021	-1.6358
JUND	Jun D proto-oncogene	1.3287	-1.5052
KCNA5	Potassium voltage-gated channel, shaker-related subfamily, member 5	1.5583	-1.057
LDHA	Lactate dehydrogenase A	1.3104	1.7532
MAF	V-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)	1.5157	2.0994
MIF	Macrophage migration inhibitory factor (glycosylation-inhibiting factor)	1.5583	1.8025
NCAM1	Neural cell adhesion molecule 1	1.6818	2.2658
NF1	Neurofibromin 1	1.5369	1.6472
NOS2	Nitric oxide synthase 2, inducible	1.0425	1.3287
NPY	Neuropeptide Y	-1.181	1.2746
NR4A2	Nuclear receptor subfamily 4, group A, member 2	1.1329	1.6358
PCK2	Phosphoenolpyruvate carboxykinase 2 (mitochondrial)	1.057	-1.0792
PCNA	Proliferating cell nuclear antigen	1.0867	1.014
PENK	Proenkephalin	1.057	-1.0497
PER1	Period homolog 1 (Drosophila)	1.4241	-1.8404
PLAT	Plasminogen activator, tissue	1.5052	1.0425
PLN	Phospholamban	1.1019	1.815
PMAIP1	Phorbol-12-myristate-13-acetate-induced protein 1	1.4845	1.5476
POU1F1	POU class 1 homeobox 1	1.5911	1.1019
POU2AF1	POU class 2 associating factor 1	1.3472	-1.3013

Table A-2. (Continued)

Gene	Description	AC2	AC6
PPP1R15A	Protein phosphatase 1, regulatory (inhibitor) subunit 15A	1.0497	-1.4948
PPP2CA	Protein phosphatase 2, catalytic subunit, alpha isozyme	-1	1.2746
PRKAR1A	Protein kinase, cAMP-dependent, regulatory, type I, alpha (tissue specific extinguisher 1)	1.3195	-1.0425
PRL	Prolactin	1.5583	-1.057
PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	1.1251	1.3947
RB1	Retinoblastoma 1	1.1975	1.2924
S100A12	S100 calcium binding protein A12	1.5583	-1.057
S100A6	S100 calcium binding protein A6	1.5583	1.0943
S100G	S100 calcium binding protein G	3.8637	2.7511
SCG2	Secretogranin II	-1.2483	1.4044
SGK1	Serum/glucocorticoid regulated kinase 1	1.0497	1.9053
SLC18A1	Solute carrier family 18 (vesicular monoamine), member 1	1.5583	-1.057
SOD2	Superoxide dismutase 2, mitochondrial	1.1251	1.1728
SRF	Serum response factor (c-fos serum response element-binding transcription factor)	1.2226	-1.1567
SST	Somatostatin	1.5583	-1.057
SSTR2	Somatostatin receptor 2	1.6586	1.434
STAT3	Signal transducer and activator of transcription 3 (acute-phase response factor)	1.0353	1.257
TACR1	Tachykinin receptor 1	2.042	-1.1096
TGFB3	Transforming growth factor, beta 3	1.0943	-1.366
TH	Tyrosine hydroxylase	1.5583	-1.057
THBS1	Thrombospondin 1	1.2226	1.5583
TNF	Tumor necrosis factor	1.5692	4.8568
VCL	Vinculin	1.1173	1.4641
VIP	Vasoactive intestinal peptide	1.8921	4.4691

Notes: HEK-293 were treated with 1 μ M Fsk for 24 h. Gene expression is expressed as fold change with respect to empty pEGFP-n1 vector.

Table A-3. GPCR expression in BSMC

Gene	Description	Average CP	SD
LPAR1	Lysophosphatidic acid receptor 1	23.49	0.30
F2R	Coagulation factor II (thrombin) receptor	23.58	0.37
GPR176	G protein-coupled receptor 176	24.41	0.10
PDGFRB	Platelet-derived growth factor receptor, beta polypeptide	24.76	0.24
F2RL2	Coagulation factor II (thrombin) receptor-like 2	24.92	0.40
SIGMAR1	Sigma non-opioid intracellular receptor 1	25.06	0.21
FZD1	Frizzled family receptor 1	25.40	0.30
CCRL1	Chemokine (C-C motif) receptor-like 1	25.82	0.23
NPR2	Natriuretic peptide receptor B/guanylate cyclase B (atrionatriuretic peptide receptor B)	25.82	0.18
TM2D1	TM2 domain containing 1	25.85	0.28
EDNRB	Endothelin receptor type B	25.90	0.25
FZD6	Frizzled family receptor 6	26.00	0.14
LPHN2	Latrophilin 2	26.01	0.18
GPRC5A	G protein-coupled receptor, family C, group 5, member A	26.02	0.36
CD97	CD97 molecule	26.06	0.33
OPN3	Opsin 3	26.06	0.13
NPR3	Natriuretic peptide receptor C/guanylate cyclase C (atrionatriuretic peptide receptor C)	26.13	0.13
FZD7	Frizzled family receptor 7	26.29	0.51
S1PR2	Sphingosine-1-phosphate receptor 2	26.38	0.17
OPN1SW	Opsin 1 (cone pigments), short-wave-sensitive	26.39	0.08
EDNRA	Endothelin receptor type A	26.44	0.29
CHRM2	Cholinergic receptor, muscarinic 2	26.49	0.42
S1PR3	Sphingosine-1-phosphate receptor 3	26.53	0.11
BDKRB1	Bradykinin receptor B1	26.53	0.11
FZD2	Frizzled family receptor 2	26.61	0.06
BDKRB2	Bradykinin receptor B2	26.66	0.18
XPR1	Xenotropic and polytropic retrovirus receptor 1	26.74	0.37
GPR157	G protein-coupled receptor 157	27.04	0.11
CRCP	CGRP receptor component	27.17	0.13
ELTD1	EGF, latrophilin and seven transmembrane domain containing 1	27.29	0.24

Table A-3. (Continued)

Gene	Description	Average CP	SD
OGFR	Opioid growth factor receptor	27.36	0.39
GPR153	G protein-coupled receptor 153	27.51	0.51
FZD4	Frizzled family receptor 4	27.54	0.35
ADORA2 B	Adenosine A2b receptor	27.61	0.67
GPR125	G protein-coupled receptor 125	27.62	0.47
SSTR1	Somatostatin receptor 1	27.62	0.10
GPR124	G protein-coupled receptor 124	27.68	0.02
PDGFR	Platelet-derived growth factor receptor-like	27.71	0.66
PTGER4	Prostaglandin E receptor 4 (subtype EP4)	27.71	0.31
LEPR	Leptin receptor	27.74	0.22
MRGPRF	MAS-related GPR, member F	27.78	0.45
MRGPR G	MAS-related GPR, member G	27.85	0.91
S1PR1	Sphingosine-1-phosphate receptor 1	27.99	0.94
NTSR1	Neurotensin receptor 1 (high affinity)	28.20	0.32
FZD8	Frizzled family receptor 8	28.24	0.46
HRH1	Histamine receptor H1	28.32	0.45
CHRM1	Cholinergic receptor, muscarinic 1	28.34	0.83
PTGFR	Prostaglandin F receptor (FP)	28.38	0.25
GPRC5B	G protein-coupled receptor, family C, group 5, member B	28.39	0.13
GPR161	G protein-coupled receptor 161	28.48	0.06
GPR37	G protein-coupled receptor 37 (endothelin receptor type B-like)	28.54	0.06
GPR152	G protein-coupled receptor 152	28.60	0.04
F2RL1	Coagulation factor II (thrombin) receptor-like 1	28.60	0.08
GPR27	G protein-coupled receptor 27	28.64	0.17
GPR85	G protein-coupled receptor 85	28.65	0.16
GPR39	G protein-coupled receptor 39	28.66	0.23
LPAR3	Lysophosphatidic acid receptor 3	28.67	0.25
CNR2	Cannabinoid receptor 2 (macrophage)	28.82	0.71
SMO	Smoothed, frizzled family receptor	28.86	0.40
CXCR7	Chemokine (C-X-C motif) receptor 7	29.00	0.97
GPR135	G protein-coupled receptor 135	29.03	0.04
LPAR6	Lysophosphatidic acid receptor 6	29.04	0.01
GPR173	G protein-coupled receptor 173	29.04	0.20

Table A-3. (Continued)

Gene	Description	Average CP	SD
GPR1	G protein-coupled receptor 1	29.10	0.08
P2RY11	Purinergic receptor P2Y, G-protein coupled, 11	29.13	0.03
OPRD1	Opioid receptor, delta 1	29.29	1.06
CASR	Calcium-sensing receptor	29.36	1.24
SSTR4	Somatostatin receptor 4	29.38	0.59
GPR31	G protein-coupled receptor 31	29.56	1.34
ADRA1D	Adrenergic, alpha-1D-, receptor	29.62	0.64
GPR162	G protein-coupled receptor 162	29.64	0.16
GABBR1	Gamma-aminobutyric acid (GABA) B receptor, 1	29.65	0.03
F2RL3	Coagulation factor II (thrombin) receptor-like 3	29.71	0.78
GPR75	G protein-coupled receptor 75	29.78	0.25
LPHN1	Latrophilin 1	29.85	0.18
ADRB2	Adrenergic, beta-2-, receptor, surface	29.90	0.56
GPR37L1	G protein-coupled receptor 37 like 1	29.96	0.52
GPR68	G protein-coupled receptor 68	30.09	0.35
NPR1	Natriuretic peptide receptor A/guanylate cyclase A (atrionatriuretic peptide receptor A)	30.15	0.77
HTR7	5-hydroxytryptamine (serotonin) receptor 7 (adenylate cyclase-coupled)	30.17	0.91
CHRM4	Cholinergic receptor, muscarinic 4	30.22	0.46
GPR12	G protein-coupled receptor 12	30.23	0.11
BAI2	Brain-specific angiogenesis inhibitor 2	30.26	0.26
GPR55	G protein-coupled receptor 55	30.37	0.07
CCBP2	Chemokine binding protein 2	30.38	1.15
GPR126	G protein-coupled receptor 126	30.40	0.41
GPR111	G protein-coupled receptor 111	30.53	0.71
GPR97	G protein-coupled receptor 97	30.56	0.47
LPAR5	Lysophosphatidic acid receptor 5	30.61	0.18
MRGPRD	MAS-related GPR, member D	30.65	0.22
TBXA2R	Thromboxane A2 receptor	30.66	0.12
CCR3	Chemokine (C-C motif) receptor 3	30.68	0.62
GPR63	G protein-coupled receptor 63	30.70	0.16
PTGIR	Prostaglandin I2 (prostacyclin) receptor (IP)	30.73	0.17
NTSR2	Neurotensin receptor 2	30.76	0.08
GPR78	G protein-coupled receptor 78	30.83	0.34
HTR3A	5-hydroxytryptamine (serotonin) receptor 3A	31.12	0.91

Table A-3. (Continued)

Gene	Description	Average CP	SD
FZD5	Frizzled family receptor 5	31.18	1.01
GPR56	G protein-coupled receptor 56	31.20	0.13
GPR3	G protein-coupled receptor 3	31.26	0.35
DRD4	Dopamine receptor D4	31.28	0.41
PTGER3	Prostaglandin E receptor 3 (subtype EP3)	31.28	0.03
RXFP3	Relaxin/insulin-like family peptide receptor 3	31.29	0.37
CCR7	Chemokine (C-C motif) receptor 7	31.33	0.18
NMUR1	Neuromedin U receptor 1	31.34	0.74
TAAR5	Trace amine associated receptor 5	31.35	0.48
MRGPRE	MAS-related GPR, member E	31.37	0.12
LTB4R	Leukotriene B4 receptor	31.40	0.20
GPR26	G protein-coupled receptor 26	31.49	0.08
GPR65	G protein-coupled receptor 65	31.51	0.71
GPR44	G protein-coupled receptor 44	31.59	0.18
AVPR1A	Arginine vasopressin receptor 1A	31.63	0.23
GPR182	G protein-coupled receptor 182	31.63	0.28
CRHR1	Corticotropin releasing hormone receptor 1	31.64	0.16
CALCRL	Calcitonin receptor-like	31.76	0.08
GNRHR	Gonadotropin-releasing hormone receptor	31.76	0.74
XCR1	Chemokine (C motif) receptor 1	31.79	0.19
CX3CR1	Chemokine (C-X3-C motif) receptor 1	31.79	0.34
GPR160	G protein-coupled receptor 160	31.81	0.22
RHO	Rhodopsin	31.81	0.54
CCR10	Chemokine (C-C motif) receptor 10	31.82	0.04
PTGER1	Prostaglandin E receptor 1 (subtype EP1), 42kDa	31.82	0.71
VIPR2	Vasoactive intestinal peptide receptor 2	31.83	0.06
GPR133	G protein-coupled receptor 133	31.86	0.18
NMUR2	Neuromedin U receptor 2	31.87	0.42
NPSR1	Neuropeptide S receptor 1	31.87	0.88
GPRC5D	G protein-coupled receptor, family C, group 5, member D	31.88	0.38
NPBWR1	Neuropeptides B/W receptor 1	31.91	0.30
P2RY2	Purinergic receptor P2Y, G-protein coupled, 2	31.94	0.99
CELSR3	Cadherin, EGF LAG seven-pass G-type receptor 3 (flamingo homolog, Drosophila)	31.99	0.06
ADORA1	Adenosine A1 receptor	32.01	0.30

Table A-3. (Continued)

Gene	Description	Average CP	SD
OPRK1	Opioid receptor, kappa 1	32.02	0.28
CXCR6	Chemokine (C-X-C motif) receptor 6	32.06	0.37
CELSR2	Cadherin, EGF LAG seven-pass G-type receptor 2 (flamingo homolog, Drosophila)	32.07	0.11
ADRA1A	Adrenergic, alpha-1A-, receptor	32.09	0.51
GPR179	G protein-coupled receptor 179	32.12	0.11
P2RY6	Pyrimidinergic receptor P2Y, G-protein coupled, 6	32.20	0.37
S1PR5	Sphingosine-1-phosphate receptor 5	32.26	0.42
GABBR2	Gamma-aminobutyric acid (GABA) B receptor, 2	32.29	0.95
ADRA2A	Adrenergic, alpha-2A-, receptor	32.32	0.57
GPR174	G protein-coupled receptor 174	32.32	0.17
GPR35	G protein-coupled receptor 35	32.33	0.68
GPR62	G protein-coupled receptor 62	32.34	0.40
ADRA1B	Adrenergic, alpha-1B-, receptor	32.35	0.54
GPR183	G protein-coupled receptor 183	32.39	0.11
GPR21	G protein-coupled receptor 21	32.41	0.11
OPRM1	Opioid receptor, mu 1	32.42	0.43
CCR4	Chemokine (C-C motif) receptor 4	32.45	0.51
GPR142	G protein-coupled receptor 142	32.45	0.25
ADORA2A	Adenosine A2a receptor	32.47	0.01
HTR1B	5-hydroxytryptamine (serotonin) receptor 1B	32.48	0.89
GPR18	G protein-coupled receptor 18	32.54	0.50
MTNR1A	Melatonin receptor 1A	32.56	0.01
PPYR1	Pancreatic polypeptide receptor 1	32.59	0.04
LTB4R2	Leukotriene B4 receptor 2	32.61	0.16
SORCS1	Sortilin-related VPS10 domain containing receptor 1	32.65	0.28
P2RY14	Purinergic receptor P2Y, G-protein coupled, 14	32.68	0.76
GPR45	G protein-coupled receptor 45	32.72	1.00
TACR3	Tachykinin receptor 3	32.75	0.06
GPR101	G protein-coupled receptor 101	32.81	0.15
GPR64	G protein-coupled receptor 64	32.92	0.84
PTGDR	Prostaglandin D2 receptor (DP)	32.92	1.05
GPR52	G protein-coupled receptor 52	32.93	0.35
PTH1R	Parathyroid hormone 1 receptor	32.94	0.09

Table A-3. (Continued)

Gene	Description	Average CP	SD
LPAR2	Lysophosphatidic acid receptor 2	32.95	0.47
CHRM3	Cholinergic receptor, muscarinic 3	33.01	0.69
SSTR2	Somatostatin receptor 2	33.03	1.60
GHRHR	Growth hormone releasing hormone receptor	33.06	0.15
GPR77	G protein-coupled receptor 77	33.09	0.08
CCKBR	Cholecystokinin B receptor	33.12	1.15
HCAR1	Hydroxycarboxylic acid receptor 1	33.12	0.45
NPBWR2	Neuropeptides B/W receptor 2	33.17	0.65
NPFFR1	Neuropeptide FF receptor 1	33.17	0.16
TAAR2	Trace amine associated receptor 2	33.18	1.00
GPR19	G protein-coupled receptor 19	33.20	1.78
PTAFR	Platelet-activating factor receptor	33.28	0.05
GPR149	G protein-coupled receptor 149	33.33	1.10
PROKR2	Prokineticin receptor 2	33.33	1.58
GPR83	G protein-coupled receptor 83	33.35	0.29
TRHR	Thyrotropin-releasing hormone receptor	33.39	0.86
ADRB1	Adrenergic, beta-1-, receptor	33.39	0.81
GPR32	G protein-coupled receptor 32	33.45	0.32
AVPR2	Arginine vasopressin receptor 2	33.55	0.15
GPR115	G protein-coupled receptor 115	33.59	0.69
GALR2	GALANIN RECEPTOR 2	33.61	0.72
FZD9	Frizzled family receptor 9	33.63	0.95
GPR148	G protein-coupled receptor 148	33.66	0.16
HCAR2	Hydroxycarboxylic acid receptor 2	33.66	0.88
FFAR2	Free fatty acid receptor 2	33.68	0.64
MC5R	Melanocortin 5 receptor	33.69	1.29
LPAR4	Lysophosphatidic acid receptor 4	33.72	1.12
GPR113	G protein-coupled receptor 113	33.73	0.32
GPR61	G protein-coupled receptor 61	33.86	0.42
GPR144	G protein-coupled receptor 144	33.87	0.60
P2RY13	Purinergic receptor P2Y, G-protein coupled, 13	33.92	0.27
LPHN3	Latrophilin 3	33.94	0.25
S1PR4	Sphingosine-1-phosphate receptor 4	33.94	0.70
HTR1F	5-hydroxytryptamine (serotonin) receptor 1F	33.96	1.01
GPR34	G protein-coupled receptor 34	34.01	0.09
GPR82	G protein-coupled receptor 82	34.14	0.64
DRD2	Dopamine receptor D2	34.14	0.71

Table A-3. (Continued)

Gene	Description	Average CP	SD
O3FAR1	Omega-3 fatty acid receptor 1	34.15	0.04
MCHR1	Melanin-concentrating hormone receptor 1	34.24	0.77
GPR84	G protein-coupled receptor 84	34.28	0.67
P2RY4	Pyrimidinergic receptor P2Y, G-protein coupled, 4	34.29	0.97
FPR1	Formyl peptide receptor 1	34.37	0.52
RGR	Retinal G protein coupled receptor	34.42	1.29
P2RY1	Purinergic receptor P2Y, G-protein coupled, 1	34.49	0.73
TACR1	Tachykinin receptor 1	34.49	1.94
APLNR	Apelin receptor	34.67	1.26
GCGR	Glucagon receptor	34.68	0.04
GPR6	G protein-coupled receptor 6	34.81	0.23
LGR5	Leucine-rich repeat containing G protein-coupled receptor 5	34.87	0.93
TAAR9	Trace amine associated receptor 9 (gene/pseudogene)	34.87	0.13
GPBAR1	G protein-coupled bile acid receptor 1	34.89	2.65
BRS3	Bombesin-like receptor 3	34.89	0.83
GPRC6A	G protein-coupled receptor, family C, group 6, member A	35.08	1.90
GRM2	Glutamate receptor, metabotropic 2	35.09	1.12
GPR116	G protein-coupled receptor 116	35.10	0.33
GRM7	Glutamate receptor, metabotropic 7	35.11	0.30
GRM1	Glutamate receptor, metabotropic 1	35.12	0.96
GPR17	G protein-coupled receptor 17	35.22	0.16
C5AR1	Complement component 5a receptor 1	35.26	0.31
CCR8	Chemokine (C-C motif) receptor 8	35.37	1.27
RRH	Retinal pigment epithelium-derived rhodopsin homolog	35.38	1.25
FZD3	Frizzled family receptor 3	35.38	1.05
GPR119	G protein-coupled receptor 119	35.42	0.51
CCR9	Chemokine (C-C motif) receptor 9	35.44	1.12
GPR151	G protein-coupled receptor 151	35.44	0.58
GPR22	G protein-coupled receptor 22	35.45	4.15
CCR6	Chemokine (C-C motif) receptor 6	35.57	1.35
GPR156	G protein-coupled receptor 156	35.60	1.63
HTR6	5-hydroxytryptamine (serotonin) receptor 6	35.64	0.76
DARC	Duffy blood group, chemokine receptor	35.66	1.05
GPR123	G protein-coupled receptor 123	35.66	0.14

Table A-3. (Continued)

Gene	Description	Average CP	SD
NPFFR2	Neuropeptide FF receptor 2	35.68	0.19
CXCR5	Chemokine (C-X-C motif) receptor 5	35.74	1.33
CMKLR1	CHEMOKINE-LIKE RECEPTOR 1	35.97	1.71
TSHR	Thyroid stimulating hormone receptor	36.04	0.53
CALCR	CALCITONIN RECEPTOR	36.08	0.51
BAI1	Brain-specific angiogenesis inhibitor 1	36.23	0.78
GPR171	G protein-coupled receptor 171	36.24	2.38
GRIK3	Glutamate receptor, ionotropic, kainate 3	36.26	0.05
NPY1R	Neuropeptide Y receptor Y1	36.30	0.52
GPR15	G protein-coupled receptor 15	36.31	0.40
TAAR6	Trace amine associated receptor 6	36.40	0.64
CXCR4	Chemokine (C-X-C motif) receptor 4	36.42	0.36
PRLHR	Prolactin releasing hormone receptor	36.42	1.16
MC4R	Melanocortin 4 receptor	36.58	0.16
GPR112	G protein-coupled receptor 112	36.60	0.06
GPR139	G protein-coupled receptor 139	36.60	0.25
OPN5	Opsin 5	36.64	0.62
GPR50	G protein-coupled receptor 50	36.66	0.23
NPY2R	Neuropeptide Y receptor Y2	36.70	0.04
GPR87	G protein-coupled receptor 87	36.70	2.73
GLP2R	Glucagon-like peptide 2 receptor	36.79	0.76
OXGR1	Oxoglutarate (alpha-ketoglutarate) receptor 1	36.97	0.08
CXCR2	Chemokine (C-X-C motif) receptor 2	37.03	1.14
PROKR1	Prokineticin receptor 1	37.06	0.43
P2RY10	Purinergic receptor P2Y, G-protein coupled, 10	37.21	0.60
GPR128	G protein-coupled receptor 128	37.24	0.11
GALR1	Galanin receptor 1	37.34	3.77
GPR143	G protein-coupled receptor 143	37.53	1.32
HRH4	Histamine receptor H4	37.53	3.49
SSTR3	Somatostatin receptor 3	37.56	0.65
TAAR1	Trace amine associated receptor 1	37.66	0.49
MRGPRX4	MAS-related GPR, member X4	37.69	2.13
HTR2C	5-hydroxytryptamine (serotonin) receptor 2C	37.82	0.30
ADCYAP1R1	Adenylate cyclase activating polypeptide 1 (pituitary) receptor type I	37.69	0.64
HTR2A	5-hydroxytryptamine (serotonin) receptor 2A	37.83	3.07
FZD10	Frizzled family receptor 10	38.04	0.27

Table A-3. (Continued)

Gene	Description	Average CP	SD
SORCS3	Sortilin-related VPS10 domain containing receptor 3	38.10	2.69
MRGPRX3	MAS-related GPR, member X3	38.48	2.16
GRM8	Glutamate receptor, metabotropic 8	38.52	0.54
ADORA3	Adenosine A3 receptor	38.79	1.72
MRGPRX2	MAS-related GPR, member X2	38.86	1.61
CYSLTR1	Cysteinyl leukotriene receptor 1	38.99	1.44
HTR4	5-hydroxytryptamine (serotonin) receptor 4	39.02	1.39
SCTR	Secretin receptor	39.07	1.32
GPR141	G protein-coupled receptor 141	39.27	1.04
MC2R	Melanocortin 2 receptor (adrenocorticotrophic hormone)	39.52	0.69
HTR1A	5-hydroxytryptamine (serotonin) receptor 1A	39.70	0.42
AGTR2	Angiotensin II receptor, type 2	40.00	0.00
BAI3	Brain-specific angiogenesis inhibitor 3	40.00	0.00
CCRL2	Chemokine (C-C motif) receptor-like 2	40.00	0.00
CYSLTR2	Cysteinyl leukotriene receptor 2	40.00	0.00
DRD5	Dopamine receptor D5	40.00	0.00
GALR3	Galanin receptor 3	40.00	0.00
GRM5	Glutamate receptor, metabotropic 5	40.00	0.00
HCRTR1	Hypocretin (orexin) receptor 1	40.00	0.00
LHCGR	Luteinizing hormone/choriogonadotropin receptor	40.00	0.00
OPN4	Opsin 4	40.00	0.00
P2RY12	Purinergic receptor P2Y, G-protein coupled, 12	40.00	0.00
SUCNR1	Succinate receptor 1	40.00	0.00
QRFPR	Pyroglutamylated RFamide peptide receptor	40.00	0.00

VITA

Amy Bogard was born in Memphis, TN in 1984. She was raised in York, PA and Jackson, TN. Amy earned a Bachelor of Science in Biology from the University of Tennessee Chattanooga in 2008. In August 2008 she entered the Integrated Program in Biomedical Sciences (IPBS) at the University of Tennessee Health Science Center under the Cell Signaling and Molecular Therapeutics track and in 2009 joined the laboratory of Dr. Rennolds Ostrom in the Department of Pharmacology. She is expected to earn her Ph.D. in December 2013.