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## Role of an Adenylyl Cyclase Isoform in Alcohol's Effect on Cyclic AMP Regulated Gene Expression in Mammalian Cells

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ROLE OF AN ADENYLYL CYCLASE ISOFORM IN ALCOHOL'S EFFECT ON  
CYCLIC AMP REGULATED GENE EXPRESSION IN MAMMALIAN CELLS

A Dissertation

Submitted to the Graduate Faculty of the  
Louisiana State University and  
Agricultural and Mechanical College  
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in

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by

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## ABSTRACT

Research suggests that the cyclic AMP (cAMP) signaling pathway including CREB-CRE regulated expression of various genes is implicated in the predisposition to and development of alcoholism in humans. Alcohol also induces changes in inflammatory and immune responses; these changes increase the incidence of pneumonias and other infections, which can negatively affect recovery from infections. Cyclic AMP (cAMP) is known for its immunosuppressive effects and is also required for proper development of the immune system. Previous work in our laboratory has demonstrated that ethanol enhances the activity of adenylyl cyclase (AC) in an isoform-specific manner; type 7 AC (AC7) is most enhanced by ethanol. Therefore, we hypothesize that the AC isoform expressed in the cells will play a role in ethanol's effects on cAMP regulated gene expression. We further hypothesize that alcohol modulates cAMP signaling in immune cells by enhancing the activity of AC7; thus, AC7 may play a role in ethanol's effects on immune function. Our objectives include: 1) evaluate the AC isoform specific effects of ethanol on cAMP regulated gene in NIH 3T3 cells by overexpressing two AC isoforms: AC3 and AC7; 2) employ immune cell lines endogenously expressing AC7, RAW 264.7 and BV-2, to further elucidate the role of AC7 in the effect of ethanol on cAMP regulated gene expression. To examine these objectives, time-lapse fluorescent resonance energy transfer (FRET) and cAMP accumulation assays were used to monitor cAMP levels within the cells. A reporter gene (luciferase) driven by an artificial promoter inducible with cAMP was utilized to evaluate the effect of ethanol on cAMP regulated gene expression. CREB phosphorylation and nuclear translocation of transducers of regulated CREB (TORCs) were examined by western blotting. Stimulation of AC activity by the addition of dopamine caused an increase in the reporter gene activity. Ethanol potentiated the increase of reporter gene activity in NIH 3T3 cells

expressing AC7, while cells expressing AC3 did not respond to ethanol. Cyclic AMP pathway activation via stimulation with prostaglandin E1 (PGE<sub>1</sub>) showed an increase in cAMP and reporter gene expression in RAW 264.7 and BV-2 cells. The effect observed was potentiated in the presence of ethanol. Cyclic AMP analog, 8-Bromo-cAMP, induced luciferase activity was not significantly affected by ethanol. The level of CREB phosphorylation did not change by cAMP stimulation or in the presence of ethanol. However, there were significant changes in the TORC3 amount in nuclei depending on stimulation conditions. The results suggest that nuclear translocation of TORC3 may play a more critical role than CREB phosphorylation in the observed changes in the cAMP driven reporter gene activity. Furthermore, the ethanol effect on cAMP regulated reporter gene expression is due to a change in the amount of cAMP, which most likely results from the enhancement of AC7 activity by ethanol.



## **CHAPTER 1. INTRODUCTION**

### **Alcohol Use and Abuse**

Alcohol use in the United States and in most countries around the world is very common. The consumption of alcoholic beverages in excess has a global socio-economic cost and is considered to be a significant health risk for injury and chronic disease (Rehm et al., 2009). The effects of alcohol use vary greatly; many individuals can drink alcohol without developing problematic symptoms, while others develop long term problems and pathologies. There is also evidence suggesting that moderate alcohol use may be beneficial. To address the health implications of alcohol use in the United States, The National Institute on Alcohol Abuse and Alcoholism (NIAAA) was established. As of 2016, the NIAAA yearly budget was approximately 450 million dollars. According to the NIAAA, over 56% of US adults age 18 and above are regular drinkers (National Institute on Alcohol Abuse and Alcoholism, 2016). A regular drinker is defined as someone who consumes 12 or more drinks per year; one drink is defined as 12 ounces of beer, 5 ounces of wine, or 1.5 ounces of 80-proof liquor. Per capita ethanol consumption was estimated at 2.33 gallons in 2012. That year, more than 1.2 million drivers were arrested for driving under the influence. According to the Centers for Disease Control and Prevention, 10,076 people were killed in alcohol-impaired driving crashes in 2013; this statistic accounted for nearly one-third of all traffic related deaths in the United States that year. The overall economic cost of alcohol abuse to the United States is estimated to be \$249 billion annually (Sacks et al., 2010). This figure is primarily attributed to health care costs related to excessive drinking, crime, and reduced work productivity.

Alcohol abuse and alcohol dependence are classified as alcohol use disorders (AUD) and are sub-categorized as mild, moderate, and severe. Under the Diagnostic and Statistical Manual

of Mental Disorders, Fifth Edition (DSM-V), anyone meeting two of the 11 criteria during the same 12-month period would receive a diagnosis of mild AUD; meeting four of the 11 criteria would lead to a moderate AUD; meeting six or more of the 11 criteria would lead to a severe AUD(American Psychiatric Association, 2013). The 11 criteria include: drinking larger amounts of alcohol due to increased tolerance; inability to stop drinking or a persistent desire to drink; increased amount of time spent drinking; insatiable cravings for alcohol; failure to fulfill personal obligations due to recurrent alcohol use; continued alcohol use in spite of social or interpersonal problems; giving up important activities in order to drink; alcohol use leading to physically harmful situations; alcohol induced depressed or anxiety; the need for increased amounts of alcohol for desired effect; withdrawal symptoms caused by alcohol.

### **Alcohol and the Brain**

The effects of alcohol on the brain are complex and diverse. Many regions of the brain are affected by alcohol; these regions include the cerebellum, cerebral cortex, and limbic system. (Oscar-Berman and Marinkovic, 2003; Zahr et al., 2010). The cerebellum plays a primary role in motor function. Neuroimaging evidence suggests that the cerebellum may also be involved in cognitive functions including language, attention, and memory (Desmond and Fiez, 1998). The effect of alcohol on the cerebellum has been shown to compromise impulse control, increase disinhibition, and decrease capacity for conflict processing (Zahr et al., 2010). The cerebral cortex is responsible for emotion processing and cognitive functions; damage to the cerebral cortex can impair one's ability to learn, remember, and solve problems. Similar to the cerebellum, neurodegeneration has been observed in the cerebral cortex of alcoholics (Oscar-Berman and Marinkovic, 2007).

The limbic system includes the following brain regions: thalamus, hypothalamus, hippocampus, basal ganglia, cingulate gyrus, and amygdala. These areas are responsible for regulating emotion and memory. Amygdala activation is reduced in the alcoholic brain; this deficit could lead to inadequate signaling to hypothalamic and prefrontal areas involved in emotional processing (Glahn et al., 2007). The dentate gyrus of the hippocampus produces neurons throughout a person's life span; this neurogenesis is reduced in alcoholic patients. This deficit may account for the amnesia chronic alcohol users experience (Geil et al., 2014; Oscar-Berman and Marinkovic, 2007). Chronic alcohol abuse can also result in brain atrophy. Compared to controls, postmortem analysis of alcoholic brains showed significant brain atrophy (Harper and Kril, 1985). Neuroimaging studies have also shown that alcoholics exhibit significant loss of brain matter volume, primarily in the cerebral cortex, cerebellum, and limbic system (Oscar-Berman and Marinkovic, 2003).

Chronic alcohol consumption can cause neurochemical changes. The effects of alcohol range from direct modulation of neurotransmitter systems to indirect outcomes caused by or associated with alcohol dependence. Alcohol's effects are highly dependent on the dose and exposure duration (Vengeliene et al., 2008). Nearly every neurotransmitter system in the central nervous system is affected by alcohol. The transmitters primarily involved in the process of alcohol addiction and withdraw include glutamate, glycine, gamma-aminobutyric acid (GABA), serotonin, dopamine, and opioids. These transmitters' receptors are classified as ligand-gated ion channels (LGIC), G protein coupled receptors (GPCR), or in some cases, both (Valenzuela, 1997; Vengeliene et al., 2008).

Glutamate is an excitatory neurotransmitter that acts through LGICs as well as GPRCs. Acute alcohol use inhibits glutamate release; this inhibition is partially responsible for the

sedative effects of alcohol (Lovinger, 2008). The N-methyl-D-aspartate (NMDA) receptor is a glutamate receptor that, due to a decrease in glutamate production, is also inhibited during alcohol use. Research suggests that alcohol may affect processes mediated by the NMDA receptor, including brain plasticity and excitotoxicity (Gonzales and Jaworski, 1997). Glycine is an inhibitory neurotransmitter that acts through LGICs. Glycine has been implicated in the acute and chronic effects of ethanol including tolerance, motor ataxia, impaired cognition, and sedation (Findlay et al., 2002; Perkins et al., 2010).

GABA is an inhibitory neurotransmitter. GABA<sub>A</sub> is a fast acting, ionotropic LGIC. GABA<sub>B</sub> is a slow acting, metabotropic transmembrane receptor that is a GPCR. Research suggests that short-term alcohol consumption may increase GABA<sub>A</sub> receptor function; however, long-term alcohol consumption has the opposite effect. It is suggested that the decrease in GABA<sub>A</sub> function may be caused by a decrease in receptor levels or an alteration in the receptor's subunit composition (Enoch, 2008; Lovinger, 2008; Valenzuela, 1997). Activation of the GABA<sub>B</sub> receptor suppresses alcohol intake as well as alcohol withdrawal syndrome in animals and humans. GABA<sub>B</sub> receptor activation has also reduced alcohol cravings in human patients. Based on this information, it is proposed that GABAergic drugs can modulate reward thresholds and may be a valuable tool to treat alcohol addiction and withdrawal (Agabio and Colombo, 2014; Koob, 2004; Lovinger, 2008). Serotonin primarily acts through GPCRs, but is also linked to a LGIC. Both receptor types are susceptible to alcohol. Acute alcohol exposure can enhance the signals generated by the 5-hydroxytryptamine 3 (5-HT<sub>3</sub>), LGIC receptor. This receptor resides on an axon terminal that releases GABA. Therefore, activation of the 5-HT<sub>3</sub> receptor results in a release of GABA, which increases neural inhibition (Lovinger, 2008). Several studies

have shown that pharmacological manipulations of the 5-HT system are capable of reducing alcohol consumption in animals (Vengeliene et al., 2008).

Dopamine is a neurotransmitter that only acts through GPCRs. The action of dopamine is altered by both acute and chronic alcohol exposure (Vengeliene et al., 2008). The release of dopamine is critical in alcohol reinforcement. In animal models, acute alcohol intake increases dopaminergic neuron firing and subsequent dopamine release which may reinforce the rewarding effect of alcohol consumption (Vengeliene et al., 2008). Chronic alcohol consumption can lead to suppressed dopaminergic activity. This reduction in dopamine causes the drinker to consume more alcohol in order to compensate and restore the dopamine level (Lovinger, 2008). Opioids only act through GPCRs. Opioid receptors contribute to the development of addiction in drugs including heroin and alcohol (Herz, 1997).

### **Alcohol and the Body**

Alcohol consumption has both positive and negative effects on cardiovascular health. Moderate alcohol drinkers have higher levels of high density lipoprotein cholesterol and adiponectin as well as lower levels of fibrinogen. These biomarkers indicate that moderate alcohol consumption may reduce the incidence of coronary heart disease (Brien et al., 2011). Chronic alcohol abuse can have the opposite effect on the cardiovascular system. Patients who suffer from chronic alcohol abuse have an increased risk of hypertension, cardiomyopathies, and arrhythmias (Klatsky, 2009). A recent meta-analysis of 27 studies has identified a relationship between alcohol consumption and the risk of ischemic and hemorrhagic strokes (Zhang et al., 2014). This study found no differences in the risk patterns for the two types of stroke, but found clear evidence that heavy alcohol consumption was associated with increased stroke risk.

A causal link has been established between alcohol consumption and many different types of cancer. Cancers that may be induced by alcohol consumption include: pharynx, larynx, oesophagus, liver, colon, rectum, and breast (Poschl et al., 2004). Research indicates that acetaldehyde is predominantly responsible for alcohol associated carcinogenesis. Acetaldehyde is mutagenic and carcinogenic, binds to protein and DNA, and destroys folate. Folate deficiency may further enhance alcohol-associated carcinogenesis. Additionally, methyl deficiency as a result of multiple alcohol induced changes leads to DNA hypomethylation. A depletion of folate results in the hypomethylation of oncogenes and may cause DNA strand breaks. Each of these factors have been associated with increased carcinogenesis (Mason and Choi, 2005).

### **Cyclic AMP Signaling**

Cell signaling is a means for information to be communicated between cells and their environment and allows for individual cells to respond to the needs of the organism. The majority of cell signaling cascades involve a neurotransmitter, hormone, odorant, or tastant that acts as an extracellular first messenger. This messenger, commonly referred to as a ligand, binds to a receptor located within a cell. Ligand binding to a receptor then initiates various intracellular signaling cascades. In the case of ion channel receptors, ligand binding opens an ion channel causing an efflux or influx of specific ions; ion efflux/influx changes the cells' electrical potential and can alter the concentration of a specific ion within the cell. Receptors can also be linked to other membrane bound proteins. In this case, ligand binding can initiate various intracellular events; many of these signaling cascades involve second messengers.

Cyclic adenosine monophosphate (cAMP) is a common second messenger and is involved in many biological processes including: learning and memory, fatty acid metabolism, glycolysis, myocardial contraction, cytokine production, oncogenesis, gene expression, cell

growth, and cell differentiation among others. The cAMP signaling pathway involves a G protein-coupled receptor (GPCR) linked to heterotrimeric G protein and membrane-bound adenylyl cyclase (AC). More specifically, the receptor ligand binding to the GPCR causes the receptor to undergo a conformational change; this activates  $G_s$ , a heterotrimeric G protein. The  $G_{s\alpha}$  subunits, which are part of the  $G_s$  protein, then exchange GDP for GTP. GTP-bound active  $G_{s\alpha}$  causes the dissociation of the  $G_s$  protein into  $\alpha$  and  $\beta\gamma$  subunits. Active  $G_{s\alpha}$  interacts with and activates membrane-bound AC. In addition, the  $\beta\gamma$  subunits are also capable of activating effector proteins which modify second messenger levels within a cell. G proteins mediate the stimulation or inhibition of various downstream effector enzymes and ion channels, including potassium and calcium channels, phospholipases C and A2, guanylyl cyclase, and adenylyl cyclase. Here, we will focus on the downstream effects of adenylyl cyclase stimulation, including protein kinase A (PKA) activation. Adenylyl cyclase provides the catalytic reaction that converts ATP to cAMP and is activated by  $G_{s\alpha}$ . The concentration increase of cAMP leads to the activation of cyclic nucleotide-gated ion channels, exchange protein activated by cAMP (Epac), and PKA. The activation of PKA then causes phosphorylation of various transcription factors. This pathway is deactivated by phosphodiesterase (PDE). PDEs degrade cAMP into AMP, reducing the level of intracellular cAMP (Figure 1.1) (Cooper, 2003; Strader et al., 1994).

Mammals, including humans, have nine different isoforms of membrane-bound AC, type 1 to type 9 (AC1-AC9), and one isoform of soluble AC (Cooper, 2003; Sunahara and Taussig, 2002). Each AC isoform has a distinct tissue distribution; the activity of each isoform is controlled quite differently by a variety of factors including: the  $\beta\gamma$  subunits of G proteins, calmodulin, calcium, protein kinase C (PKC), PKA, and small molecules such as magnesium and forskolin (Cooper, 2003; Sunahara and Taussig, 2002). Differential regulation of a particular AC

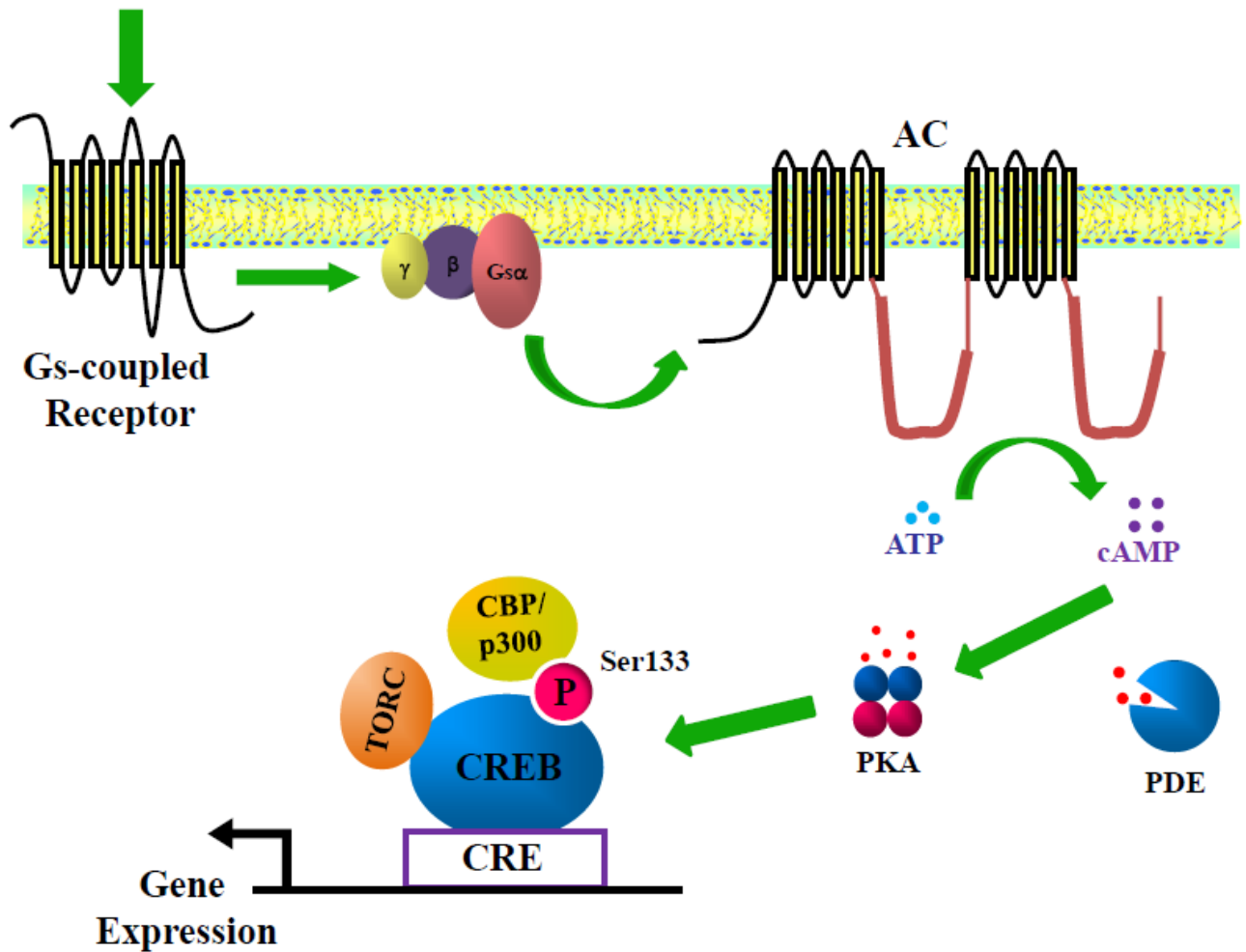


Figure 1.1. Diagram of the cAMP signaling components. Binding of a ligand to the Gs-coupled receptor activates the Gs protein which activates AC. AC converts ATP to cAMP; cAMP activates PKA. Cyclic AMP is degraded by phosphodiesterase (PDE). CREB binds to CRE. PKA activation stimulates CREB phosphorylation at Ser133; phosphorylated CREB activates CREB binding protein (CBP/p300). Activation of PKA also enhances nuclear translocation of transducers of regulated CREB (TORCs)

isoform by a stimulus allows each of the isoforms to function as a specific integrator of the external stimuli and further allows them to play an interpretive role in cAMP signal transduction. Distinct tissue distribution and regulatory profiles of the AC isoforms could render ACs important targets for a new generation of tissue- and cell-specific drugs against diseases including immune abnormalities.



G protein subunits,  $G_{\alpha}$  and  $G_{\beta\gamma}$ , have multiple isoforms capable of inhibiting or stimulating AC activity; inhibition or stimulation of an AC isoform is dependent on the characteristics of that particular isoform.  $G_{\alpha_s}$  is stimulatory to all AC isoforms;  $G_{\alpha_i}$  is inhibitory to AC1, AC5, AC6, AC8, and AC9 (Hanoune and Defer, 2001). Research has also implicated  $G_{\alpha_{12/13}}$  as being stimulatory to AC7 in particular (Jiang et al., 2008). Depending on the AC isoform, the  $G_{\beta\gamma}$  subunit can also inhibit or stimulate AC activity; typically the effect of the  $G_{\beta\gamma}$  subunit also requires  $G_{\alpha_s}$  activation (Sunahara and Taussig, 2002).

### **Alcohol and cAMP Signaling**

Numerous clinical studies have suggested that AC and the cAMP signaling system play an important role in the predisposition to and development of alcoholism (Lex et al., 1993; Menninger et al., 1998; Parsian et al., 1996; Saito et al., 1994; Tabakoff et al., 1988; Waltman et al., 1993). Abnormalities in cAMP signaling and changes in the activity of AC have also been postulated to play a vital role in the behavioral and physiological responses to alcohol in animals as well as in the development of alcoholism in humans.

Several alcohol-related parameters have been associated with cAMP signaling in experimental animal models. Ethanol-induced ataxia is correlated with cAMP accumulation in the mouse cerebellum (Kirstein and Tabakoff, 2001). Several studies have focused on the AC component of cAMP signaling in the alcohol response in animals. Double knock-out (DKO) mice lacking AC1 and AC8 had increased sensitivity to alcohol-induced sedation; DKO mice and AC8 knockout (KO) mice exhibited decreased voluntary ethanol consumption when compared to wild type mice (Maas, Jr. et al., 2005). Alcohol response has also been examined in AC5 KO mice. AC5 is highly expressed in the nucleus accumbens, a component of the brain's reward center. AC5 has also been implicated in the anxiolytic effects of alcohol. Decreased

mRNA expression of AC5 was found in the nucleus accumbens of rats one hour after treatment with ethanol (Morales-Mulia et al., 2012). Additionally, AC5 KO mice showed a higher preference for ethanol in addition to an increased total ethanol consumption compared to wild type mice. These mice also had reduced sensitivity to ethanol when exposed to ethanol-induced hypothermia and sedation via a high dose of ethanol (Kim et al., 2011). In addition, AC7 over-expression in mice has been found to increase basal and ethanol-induced DARPP-32, a downstream element of the cAMP pathway that has been implicated in the motivation for ethanol intake (Donohue et al., 2005).

Clinical research has established a link between cAMP signaling and alcoholism. Receptor-stimulated as well as basal levels of cAMP were lower in the lymphocytes of alcoholics. Lymphocytes were extracted from blood samples collected from age matched alcoholic patients and controls. Lymphocytes and isolated T cells were assayed for cAMP accumulation alone or in the presence of ethanol or an adenosine receptor agonist. The results indicated that receptor-stimulated, ethanol-stimulated, and basal cAMP levels were significantly reduced in the T cells and mixed lymphocytes from alcoholic patients (Diamond et al., 1987). Additionally, a clear reduction of ethanol-stimulated cAMP in cultured lymphocytes of alcoholic individuals has been demonstrated. Lymphocytes from control and alcoholic patients were extracted from blood samples and were propagated using cell culture for at least four generations in medium without alcohol. Compared to controls, ethanol-stimulated cAMP levels were still suppressed in the lymphocytes of alcoholics despite multiple rounds of cell division in medium not containing alcohol (Nagy et al., 1988). Subsequent research has indicated that membrane-bound adenylyl cyclase plays a role in the cAMP irregularities present in alcoholics. Subjects who abstained from alcohol but had family history positive for alcoholism generally had lower

basal and stimulated AC activity levels than complete abstainers with no family history of alcoholism (Menninger et al., 1998). Individuals with a history of alcoholism had reduced stimulated AC activity in their platelets than individuals with no history of alcoholism; these data were recorded after the individual had abstained from alcohol for at least four days. Furthermore, individuals who had consumed alcohol in the three to four days prior to platelet analysis showed higher basal and stimulated AC activity regardless of their alcoholism history (Menninger et al., 2000). This research suggests that AC activity is a possible trait marker for the diagnosis of alcohol dependence.

Our lab has previously shown that ethanol enhances the activity of AC in an isoform-specific manner (Yoshimura and Tabakoff, 1995). Additionally, the activity of AC7 can be significantly potentiated by pharmacologically relevant concentrations (less than 50 mM) of ethanol (Yoshimura and Tabakoff, 1999); these concentrations are easily attained in human blood by consuming alcoholic beverages. Alcohol cut-off is defined as the length of an *n*-alkanol carbon chain beyond which increasing the length of the carbon chain does not further increase potency of the alcohol effect. Studies using a variety of straight chain alcohols indicate that the alcohol cutoff effect for *n*-alkanol potentiation of AC activity is AC isoform specific; AC6, AC7, and AC9 have different alcohol cut-off points, likely the result of specific alcohol binding pockets on the AC protein (Kou and Yoshimura, 2007). These isoforms also vary in their response to the number and position of hydroxyl groups in a straight chain alcohol (Hasanuzzaman and Yoshimura, 2010). cAMP was monitored in real-time using a FRET based cAMP sensor; the results confirm that cAMP increases in the presence of alcohol and that the alcohol effect is AC isoform specific (Gupta et al., 2013). Based on these observations, we hypothesize that within the cAMP-generating system, AC is a main target of ethanol's action.

## **Cyclic AMP Regulated Gene Expression**

Adenylyl cyclases play an important role in cAMP-dependent activation of PKA as well as regulating cAMP Response Element Binding Protein (CREB) function via its phosphorylation. CREB mediates the activation of cAMP-responsive genes by binding as a dimer to cAMP Response Element (CRE); phosphorylated CREB then causes an increase in transcription. Changes in the activity of CREB and the expression of genes regulated by CREB have been associated with the abuse of drugs such as opiates and cocaine; these CREB regulated genes are considered to be critical to drug addiction (Chao and Nestler, 2004). Additionally, several studies have indicated that CREB is a potential ethanol target and plays a central role in the process of ethanol addiction (Chao and Nestler, 2004; Pandey et al., 2003; Pandey et al., 2004). Levels of activated CREB, which are mediated by its Ser-133 phosphorylation, are decreased during ethanol withdrawal (Pandey et al., 2001). CREB also promotes an anti-apoptotic survival signal in macrophages engaged in TLR-4 mediated immunity, which leads to enhanced host immune responses (Park et al., 2005). Ethanol enhances cAMP signaling, therefore it is hypothesized that ethanol can modulate cAMP regulated gene expression. Previous research has indicated that approximately 30% of the genes which were induced by alcohol were found to be also regulated by cAMP (Thibault et al., 2000). A new family of tissue specific and cell specific CREB co-activators known as transducers of regulated CREB activity (TORC) that bind to the bZip domain of CREB have recently been discovered (Conkright et al., 2003; Iourgenko et al., 2003). TORC1, TORC2, and TORC3 were each shown to be sequestered in the cytoplasm and translocated into the nucleus by stimulating cAMP generation and PKA in the cells (Bittinger et al., 2004).

It is well known that AC is the upstream regulator for controlling interactions between CREB and its CRE sites. A new model of the active CREB complex that is necessary for strong expression of cAMP regulated genes has been proposed to have direct interaction of TORCS with pCREB and CBP/p300. Phosphorylation of CREB is mediated by PKA. CREB phosphorylation promotes the interaction of the KID domain of CREB with the KIX domain of CBP/p300 and enhances transcription of CREB target genes (Figure 1.1) (Xu et al., 2007). Therefore, elucidation of the mechanisms through which alcohol affects cAMP regulated gene expression could lead to better treatment and/or diagnostic tools for alcoholism, alcohol abuse, and alcohol related disease.

### **Cyclic AMP and Immune Response**

It has been well established that cAMP plays a critical role in regulating immune responses (Boer et al., 2003; Castro et al., 2005; Houslay et al., 2005; Sanders, 2012). Phosphorylation by PKA in lymphocytes has been shown to regulate antigen receptor-induced signaling by altering the target protein's enzymatic activity as well as altering protein/protein interactions. Increasing intracellular cAMP in T lymphocytes inhibits the production of interleukin-2 (IL-2) and decreases lymphocyte proliferation (Averill et al., 1988; Torgensen et al., 2002). Cyclic AMP-dependent PKA has also been considered as a potential target in the therapeutic intervention of dysfunctional immune cells in autoimmune conditions including systemic lupus erythematosus as well as immunodeficiencies such as AIDS (Skålhegg et al., 2005). Cyclic AMP specific phosphodiesterase-4 (PDE4), which degrades cAMP, has been proposed as a potential target for inflammatory disease therapies including: asthma, chronic obstructive pulmonary disease, and psoriasis (Houslay et al., 2005; Theron et al., 2013). Experimental evidence suggests that  $\beta$ 2-adrenergic receptor agonists may be useful in treating

inflammation; these agonists suppress the pro-inflammatory activities of T lymphocytes, neutrophils, monocytes/macrophages, basophils, eosinophils, and mast cells via cAMP signaling in an experimental setting (Theron et al., 2013). Another important facet of cAMP signal transduction is to regulate apoptosis. While elevation of cAMP is associated with induction of apoptosis in lymphoid cells by glucocorticoids (Zhang and Insel, 2004), in many cell types including macrophages, elevated cAMP protects against apoptosis (von Knethen and Brune, 2000).

AC7 is highly expressed in the immune system and AC7 function is critical for normal immune responses (Duan et al., 2010; Jiang et al., 2013; Whiteside et al., 2011). AC7 functions to control immune system responses during bacterial infections. AC7-deficient mice display compromised antibody responses toward T cell dependent and T cell independent antigens (Duan et al., 2010). However, the role of each AC isoform, including AC7, and the effect of alcohol on immune responses has not been characterized.

### **Alcohol and Immune Response**

Alcohol alters the immune response, which can lead to increased incidence and poor outcome of infections as well as organ-specific immune-mediated damages (Szabo and Mandrekar, 2009). Acute and chronic use of alcohol can suppress the immune system via both the innate and adaptive immune response (Molina et al., 2010). Although humoral and cell-mediated immune responses are reduced in patients who consume high-doses of alcohol, studies in experimental animal models as well as humans suggest that low-doses of ethanol may enhance the immune response (Cook, 1998; Romeo et al., 2007).

Flow cytometric analysis of splenic lymphocytes has shown that chronic ethanol consumption decreases the overall number and percentage of CD4 expressing cells in rat T-

helper lymphocytes (Boyadjieva et al., 2002). Two types of CD4 T-helper lymphocytes, TH1 and TH2, exist in mice and humans (Waltenbaugh et al., 1998). TH1 responses are characterized by interleukin (IL)-12 and interferon- $\gamma$  production and function; the decrease in TH1 immunity induced by alcohol is correlated with suppressed IL-12 secretion from dendritic cells and macrophages. Acute, moderate alcohol consumption (2 ml vodka/kg body weight) may result in a dual anti-inflammatory response involving significant attenuation of IL-10, a proinflammatory cytokine produced by blood monocytes. These responses are biphasic: first, direct inhibition of NF- $\kappa$ B-mediated TNF- $\alpha$  and IL-1 $\beta$  production occurs and second, a downstream effect on IL-10 induction, resulting in secondary inflammatory cytokine production inhibition (Mandrekar et al., 2006). This research suggests that moderate alcohol use, through its anti-inflammatory effects, may counteract the activation of proinflammatory pathways.

### **Statement of the Problem and Specific Aims**

Adenylyl cyclase is a key regulator of the cAMP signaling pathway. It is well known that cAMP signaling is modulated by ethanol in humans. The effect of alcohol on AC may have downstream effects on cAMP-linked functions including CREB phosphorylation, TORC nuclear translocation, and gene expression. AC isoform specificity and alcohol's effects on cAMP regulated gene expression have not been studied. Furthermore, the effects of AC activation and alcohol on immune cells are unknown. Previous research in our lab has shown that the AC7 isoform is most enhanced by ethanol and that AC7's ethanol responsive domains reside in the cytoplasmic region of the protein. Understanding how alcohol modulates AC and subsequently gene expression may provide an explanation for many of the physiological consequences of alcohol use. We hypothesize that the AC isoform expressed in the cells dictates the effect of ethanol on cAMP regulated gene expression. AC7 is highly expressed in immune cells. Based on

this information, we further hypothesize that alcohol modulates cAMP signaling in these cells by enhancing the activity of AC7; thus, AC7 may play a role in ethanol's effects on immune function. In order to address these research problems, we have evaluated the following aims:

Aim 1: Evaluate AC isoform specificity for the effects of alcohol on cAMP regulated gene expression using NIH 3T3 cells.

Aim 2: Employ macrophage (RAW 264.7) and microglia (BV-2) cell lines to further elucidate AC7's role in ethanol's effects on cAMP regulated gene expression.

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## **CHAPTER 2.**

### **ROLE OF AN ADENYLYL CYCLASE ISOFORM IN ALCOHOL'S EFFECT ON CYCLIC AMP REGULATED REPORTER GENE EXPRESSION IN NIH 3T3 CELLS**

#### **Introduction**

Cyclic AMP (cAMP) is a second messenger molecule that plays an important regulatory role in many biological processes including lipid and sugar metabolism, cell growth, cell differentiation, learning, and memory. Cyclic AMP is generated through the activation of a membrane-bound multi-protein system, which includes adenylyl cyclase (AC), heterotrimeric G proteins, and G protein-coupled receptors (GPCR). The system is regulated by the activation of GPCRs through various extracellular stimulants including: neurotransmitters, odorants, and hormones. Previous clinical research has shown that a link between cAMP signaling and alcoholism exists in humans (Diamond et al., 1987; Lex et al., 1993; Menninger et al., 1998; Nagy et al., 1988; Tabakoff et al., 1988). Cyclic AMP signal transduction has also been postulated to play a vital role in the behavioral and physiological responses to alcohol in animals (Kim et al., 2011; Maas, Jr. et al., 2005; Moore et al., 1998). Nine membrane-bound AC isoforms, type 1 to type 9 (AC1-AC9), exist in mammals. Each AC isoform has a distinct tissue distribution, the activity of which is uniquely regulated (Cooper, 2003; Sadana and Dessauer, 2009; Sunahara and Taussig, 2002). Isoform-specific changes in expression levels have been reported for several pathological conditions: alterations of AC isoforms have been found in brains of alcoholics (Yamamoto et al., 2001), Alzheimer's disease patients (Yamamoto et al., 1996), and heroin addicts (Shichinohe et al., 2001).

Cyclase isoforms play an important role in cAMP-dependent activation of protein kinase A (PKA) as well as regulating cAMP response element binding protein (CREB) function. Studies indicate that CREB is a potential ethanol target and plays a central role in the process of

ethanol addiction (Chao and Nestler, 2004; Pandey et al., 2003; Pandey et al., 2004). Levels of activated CREB, mediated by its Ser-133 phosphorylation, are altered during ethanol withdrawal (Pandey et al., 2001). CREB mediates the activation of cAMP-responsive genes by binding as a dimer to CRE; phosphorylated CREB (pCREB) then causes an increase in transcription. Changes in the activity of CREB and the expression of genes regulated by CREB are associated with the abuse of drugs, including opiates, cocaine, and alcohol, and are critical to drug addiction (Chao and Nestler, 2004). Recently, a new family of tissue and cell specific CREB co-activators known as transducers of regulated CREB activity (TORC) that bind to the bZip domain of CREB have been discovered (Conkright et al., 2003; Iourgenko et al., 2003). TORC 1, 2, and 3 are each sequestered in the cytoplasm and translocated into the nucleus by stimulating cAMP generation (Bittinger et al., 2004). TORC1-3 also activate CRE-driven transcription; indicating that in addition to CREB, the TORC pathway may play a role in cAMP regulated gene expression. Furthermore, a new model of the active CREB complex necessary for expression of cAMP regulated genes has been proposed to have direct interaction of TORCs with pCREB and CREB binding protein (CBP/p300) (Xu et al., 2007).

Research in our laboratory has shown that ethanol enhances the activity of AC in an isoform-specific manner (Yoshimura and Tabakoff, 1995); AC7 activity can be significantly potentiated by pharmacologically relevant concentrations (less than 50 mM) of ethanol (Yoshimura and Tabakoff, 1999). These concentrations can easily be attained in human blood by consuming alcohol. In this chapter, we examine the effects of alcohol on cAMP regulated reporter gene expression. We hypothesize that AC is the main target of ethanol's action within the cAMP-generating system; ethanol's effect on cAMP signaling will be dictated by the AC isoform expressed within the cells. Thus, an alteration in cAMP inducible gene expression upon



ethanol exposure will depend on the AC isoform expressed in the cells. NIH 3T3 cells have been utilized in numerous cAMP regulated gene expression studies and were ideal for this study (Montminy, 1997). Cyclic AMP levels in NIH 3T3 cells were monitored by Epac1-camp, a fluorescent resonance energy transfer (FRET) based cAMP sensor, and a radioisotope based cAMP accumulation assay. A fusion transcription activator, GAL4-CREB, and a Firefly luciferase reporter gene driven by an artificial promoter containing GAL4 binding sites were utilized to measure changes in cAMP regulated gene expression. Renilla luciferase driven by an SV40 promoter (SV40rLuc) was used as an internal control. The changes in TORC nuclear translocation and the phosphorylation of CREB were investigated via western blotting. The results presented here provide valuable insight into the molecular mechanisms of the alcohol effect on cAMP inducible gene expression.

## **Materials and Methods**

### Reagents

Dopamine (DA), 8-bromo-cAMP (8Br-cAMP), and ethanol (EtOH) were purchased from Sigma-Aldrich (St. Louis, MO). Prostaglandin E<sub>1</sub> (PGE<sub>1</sub>) was purchased from Cayman Chemical (Ann Arbor, MI). [ $\alpha$ -<sup>32</sup>P]ATP was purchased from Perkin Elmer (Boston, MA). [2,8-<sup>3</sup>H]adenine and [8-<sup>14</sup>C]cAMP were purchased from Moravek Biochemicals (Brea, CA).

### Plasmid DNA

Plasmids containing the coding sequences for AC3 and AC7 (Yoshimura et al., 2006), Epac1-camps (Nikolaev and Lohse, 2006), GAL4-CREB (Leonard et al., 1992), p5GB/GL2 Firefly luciferase containing 5xGAL4 binding sites (Kasper et al., 1999), SV40-Renilla reporter plasmid pRL-SV40 (Promega, Madison, WI), D<sub>1A</sub> dopamine receptor (DRD1A) (Dearry et al., 1990), and green fluorescent protein (GFP) (Clontech, Mountain View, CA) were utilized.

### Cell Culture and Transfection

NIH 3T3 cells were provided by Dr. Wu Xu (Lafayette, LA). The cells were maintained in 30 ml minimal essential media (MEM) with 10% fetal bovine serum (FBS), penicillin (50  $\mu\text{g/ml}$ ), streptomycin (50  $\mu\text{g/ml}$ ), and neomycin (100  $\mu\text{g/ml}$ ) in plastic flasks (225 $\text{cm}^2$ ) at 37°C in a humidified atmosphere of 95% air and 5%  $\text{CO}_2$ . Cells were transiently transfected using Calfectin (SignaGen, Rockville, MD) according to manufacturer instructions. Transfection efficiency was monitored by observing the expression of GFP or Epac1-cAMP using an epifluorescence microscope.

### Cyclic AMP Accumulation Assay

Amounts of cAMP in the transfected NIH 3T3 cells were assessed by the cAMP accumulation assay as described previously (Hasanuzzaman and Yoshimura, 2010). Briefly, the intracellular ATP pool was labeled with 3.0  $\mu\text{Ci/ml}$  of [2,8- $^3\text{H}$ ]adenine. To determine the effect of ethanol, cells were treated with 200 mM ethanol together with 10  $\mu\text{M}$  DA for 1 minute at 37°C. The reaction was terminated by adding 50  $\mu\text{l}$  of 100% (w/v) trichloroacetic acid. ATP and cAMP contents of each well were separated through Dowex 50 and neutral alumina columns as described previously (Salomon et al., 1974) and quantified via a liquid scintillation spectrometer. [ $\alpha$ - $^{32}\text{P}$ ]ATP and [8- $^{14}\text{C}$ ]cAMP were added as internal standards in order to monitor recovery of ATP and cAMP through column chromatography.

### Fluorescence Imaging of Live Cells

A 15mm glass coverslip was coated with Poly(ethyleneimine) solution (PEI) (Sigma Aldrich, St. Louis, MO) for 30 minutes. Transfected NIH 3T3 cells were added to the coverslip 24 hours prior to the experiment. The coverslip was assembled in a perfusion chamber (~10 ml/min flow rate and 87  $\mu\text{l/mm}$  depth) attached to an eight channel perfusion valve control

system (Warner Instruments, Hamden, CT). The fluorescent imaging workstation, filter sets, and buffer composition were described previously (Gupta et al., 2013). Drugs were added for the durations indicated in the figures via perfusion. At each time point, three fluorescent images were captured using a filter set specifically for CFP, YFP, and FRET, respectively. Images were captured every 3 sec for the duration of time-lapse experiments.

### FRET Measurements

The method of sensitized FRET measurement was previously described (Sorkin et al., 2000). Background images were subtracted from the raw images prior to FRET calculations. Corrected FRET (cFRET) and normalized FRET (nFRET) were calculated as previously described (Gupta et al., 2013). nFRET values were normalized by dividing by the mean nFRET value of the first 18 time points.

### Luciferase Assay

24 hours post-transfection, cells were transferred to 24-well culture plates. Cells were treated with dopamine or 8-bromo-cAMP (1mM) in the presence or absence of ethanol. Cells were lysed and assayed for Firefly and Renilla luciferase activities using a Dual Luciferase Assay System (Promega, Madison, WI) and a Synergy HT plate reader (BioTek Instruments, Winooski, VT) according to manufacturer instructions. Results were normalized against Renilla luciferase activities obtained from the pRL-SV40 plasmid as an internal control.

### Nuclear Extract Preparation and Western Blotting

After stimulation, cells were washed with cold phosphate-buffered saline (PBS) and collected via scraping. Nuclear extracts were prepared using a NE-PER kit purchased from Pierce (Rockford, IL). Protein concentration in the extracts was determined using the BCA protein assay kit (Pierce, Rockford, IL). The nuclear extracts were stored at  $-80^{\circ}\text{C}$ . Proteins

(10 µg per lane) were separated by 12.5% SDS-PAGE and transferred to a nitrocellulose membrane. Western blotting was carried out using following primary antibodies according to manufacturer protocol: anti-CREB (Millipore, Billerica, MA), anti-phospho-CREB (pCREB) (Millipore, Billerica, MA), TORC1 (GeneTex, Irvine, CA), TORC2 (Abcam, Cambridge, MA), TORC3 (GeneTex, Irvine, CA), and β-actin (Cell Signaling, Danvers, MA). Appropriate secondary antibodies conjugated with horseradish peroxidase were obtained from Jackson ImmunoResearch (West Grove, PA). Signals were visualized by chemiluminescence using enhanced chemiluminescence (ECL) detection reagents (GE Healthcare, Piscataway, NJ), quantified using ImageJ software (Schneider et al., 2012), and normalized to β-actin for comparison.

### Statistics

Values are expressed as mean ± standard error of mean (SEM). Student's t-test, one-way ANOVA, two-way ANOVA, or two-way repeated-measures ANOVA were used to evaluate differences in values as indicated in figure legends. After ANOVA, pairwise comparisons were carried out by Holm–Sidak method. Analyses were carried out using SigmaStat (SyStat Software, San Jose, CA).

## **Results**

### Effect of Ethanol on cAMP NIH 3T3 Cells

Cyclic AMP accumulation assay using cells transfected with DRD1A clearly showed enhancement of cAMP production in the presence of dopamine. Cyclic AMP accumulation was further enhanced by dopamine in the presence of ethanol in AC7 transfected cells (Figure 2.1). Incubation without dopamine or the addition of ethanol alone did not cause any significant changes in cAMP accumulation. Cells expressing AC3 showed a similar cAMP change during 1-

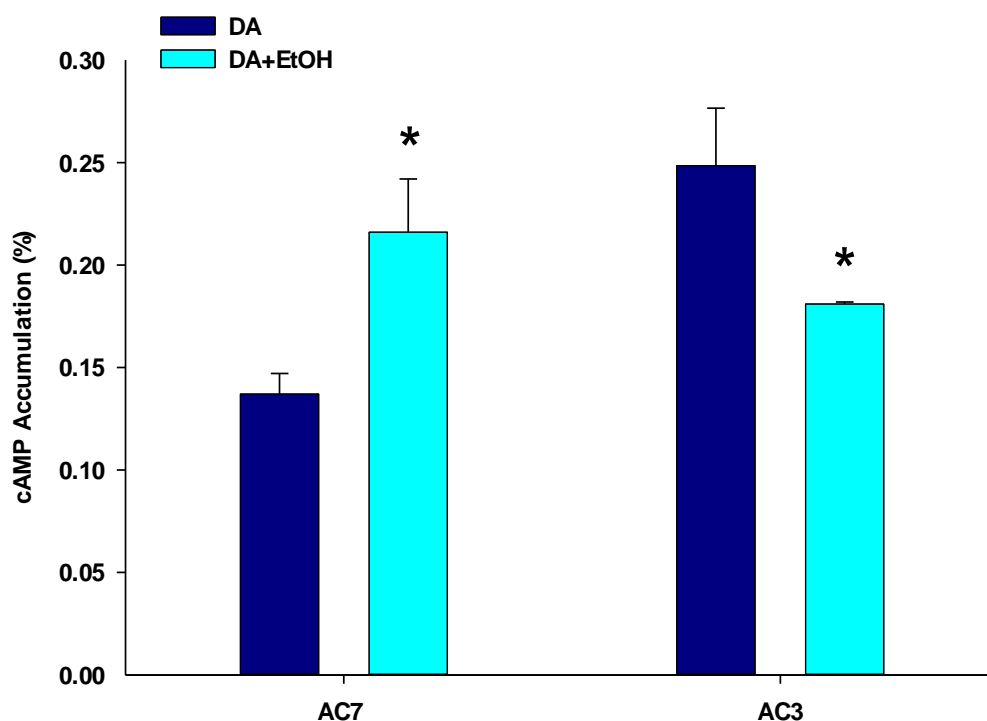


Figure 2.1. Effects of DA and EtOH on cAMP in cells expressing DRD1A and AC7 or AC3. cAMP accumulation assays were carried out in the presence of 10  $\mu$ M DA  $\pm$  200 mM EtOH for 1 minute. Percent increase in cAMP accumulation by addition of DA or DA + EtOH is displayed (n =3). \*Values significantly differ from those of DA alone (p < 0.05, student t-test).

minute stimulation with dopamine, however, dopamine in the presence of ethanol significantly decreased cAMP accumulation. The results are consistent with previous observations in which human embryonic kidney (HEK) 293 cells were utilized (Yoshimura and Tabakoff, 1995). Cells expressing Epac1-cAMP, DRD1A, and AC7 or AC3 were stimulated with dopamine in the presence or absence of ethanol for 2 minutes to monitor real-time changes in cAMP. Normalized nFRET values were plotted over time as an indicator of cAMP (Figure 2.2). In cells expressing AC7 (Figure 2.2A), nFRET rapidly decreased after the addition of dopamine. This decrease indicates an increase in cAMP. The value of nFRET reached its minimum approximately 20 seconds after addition of dopamine and remained constant during the remainder of the dopamine treatment (~100 seconds). When ethanol was added with dopamine, nFRET was further

decreased. nFRET reached its minimum ~60 seconds after addition of dopamine and ethanol. Cyclic AMP levels of the cells expressing AC3 followed a similar time course to those of cells expressing AC7 during 2-minute stimulation with DA (Figure 2.2B). However, ethanol had no significant effects on the time course or amount of cAMP in cells expressing AC3. These results are consistent with previous observation in Hela cells (Gupta et al., 2013).

#### Effect of Dopamine on cAMP Regulated Reporter Gene Expression

NIH 3T3 cells were transfected with DRD1A and AC7. Reporter gene (Firefly luciferase) activity driven by an artificial promoter inducible with cAMP showed significant enhancement during a 3 hour incubation period in the presence of dopamine (Figure 2.3). Dopamine caused a concentration-dependent increase in Firefly luciferase activity. Luciferase activity was maximized between 0.3 and 1  $\mu$ M DA.

#### Effect of Ethanol on cAMP Regulated Reporter Gene Expression

Cells expressing DRD1A and AC7 or AC3 were stimulated with dopamine and varying concentrations of ethanol for 3 hours. In cells expressing AC7, a significant increase in cAMP regulated reporter gene expression compared to dopamine alone was detected at the lowest ethanol concentration (25 mM) (Figure 2.4A). Firefly luciferase activity reached its maximum at 150 mM ethanol. In cells expressing AC3, cAMP regulated reporter gene expression was not significantly enhanced by ethanol regardless of the concentration utilized to stimulate the cells (Figure 2.4B). These results are also consistent with the isoform specific effect of ethanol on AC activity (Yoshimura and Tabakoff, 1995). Cells expressing DRD1A and AC7 or AC3 were stimulated with dopamine in the presence or absence of ethanol at varying time points (Figure 2.5). Firefly luciferase activity reached its maximum at 3 hours then slowly decreased for the duration of the experiment. In cells expressing AC7, a significant increase in cAMP regulated

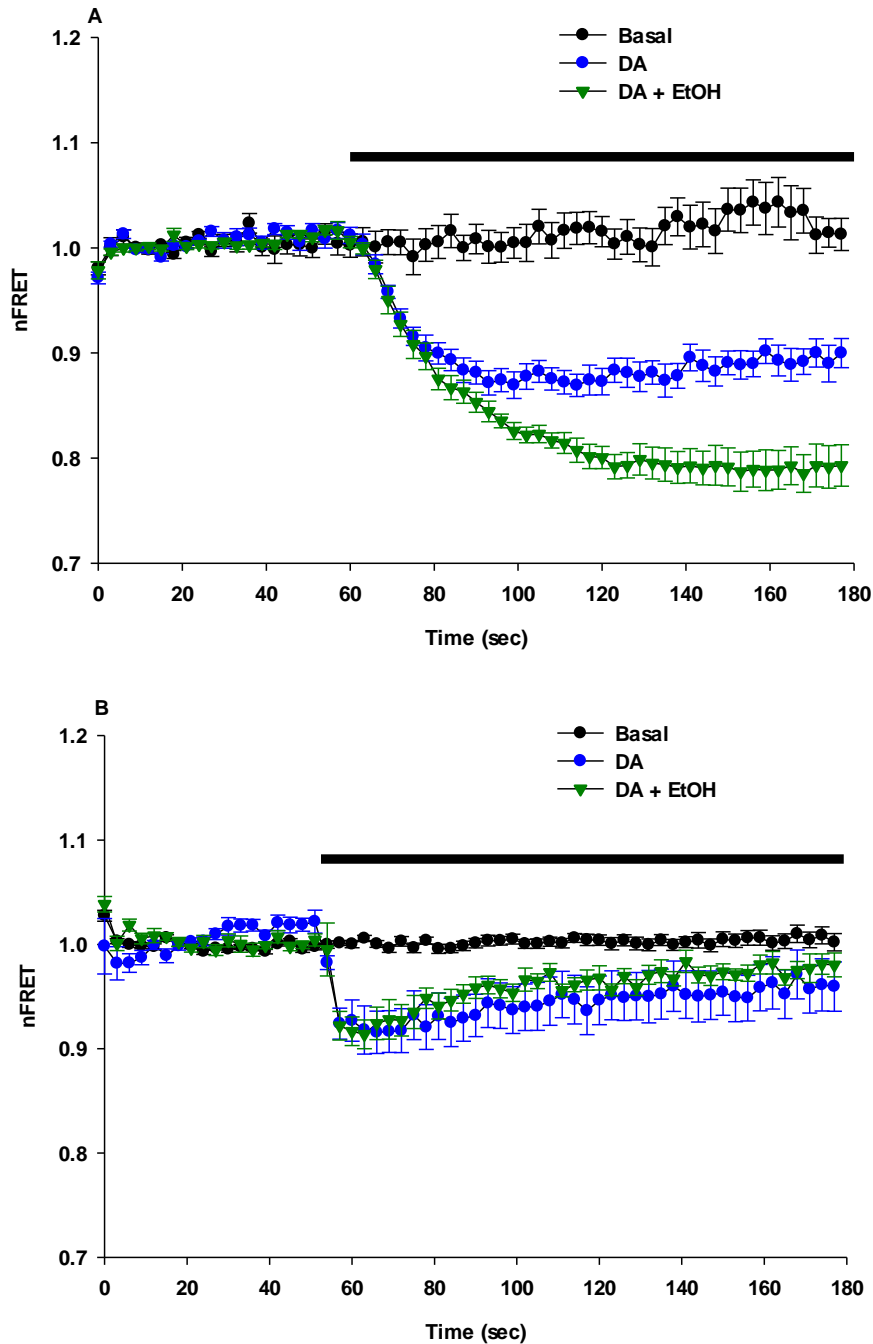


Figure 2.2. Real-time monitoring of the effects of DA and EtOH exposure on cAMP. (A) FRET-based real-time monitoring of cAMP in cells expressing AC7 and DRD1A (Basal,  $n = 8$ ; DA,  $n = 10$ ; DA + EtOH,  $n = 8$ ). nFRET values are plotted over time. The black bar indicates 2 minute duration of DA ( $3 \mu\text{M}$ ) and DA + EtOH ( $200 \text{mM}$ ) treatment. Data presented as mean  $\pm$  SEM; values past 90 seconds are significantly smaller in the presence of DA + EtOH than basal or DA alone ( $p < 0.05$ , 2-way repeated-measures ANOVA). (B) FRET-based real-time monitoring of cAMP in cells expressing AC3 and DRD1A (Basal,  $n = 13$ ; DA,  $n = 11$ ; DA + EtOH,  $n = 9$ ). Treatment and statistical analysis were similar to (A). Values past 50 seconds are significantly smaller than basal; there is no significant difference between DA alone or DA + EtOH treatment.

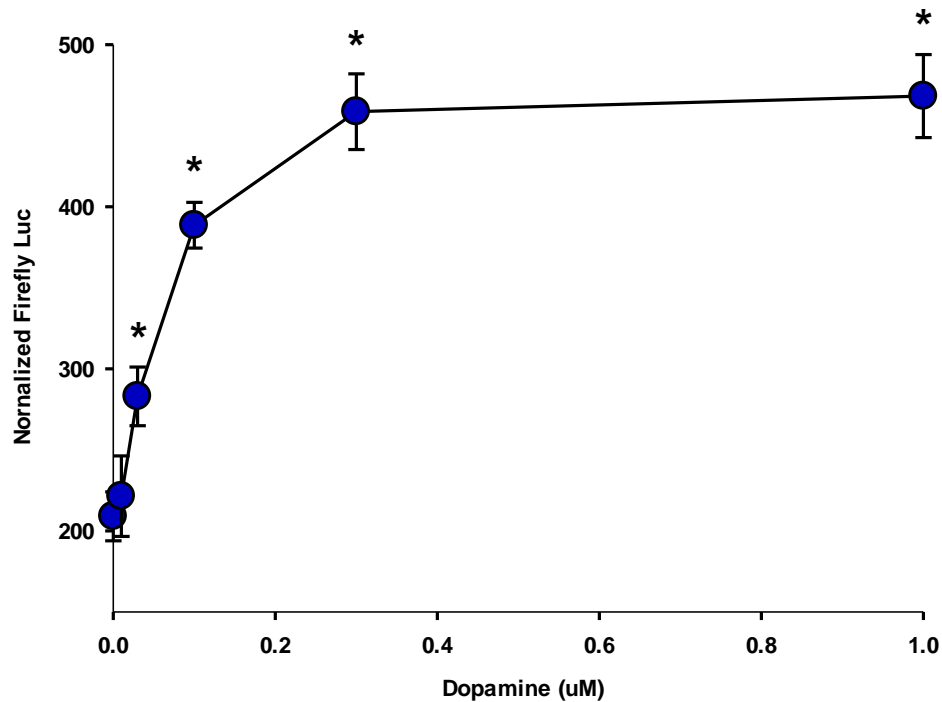


Figure 2.3. Effects of DA on cAMP stimulated reporter gene expression in cells expressing DRD1A and AC7. Cells were incubated in the presence of 0, 0.01, 0.03, 0.1, 0.3, or 1  $\mu$ M DA for 3 hours (n = 3). Basal activity in the absence of DA was  $209 \pm 15.1$ . \*Value is significantly greater than basal activity ( $p < 0.05$ , 1-way ANOVA).

reporter gene expression was detected at all time points past 2 hours by the addition of dopamine in the presence of ethanol when compared to dopamine alone (Figure 2.5A). In cells expressing AC3, cAMP regulated reporter gene expression was not significantly enhanced by DA + EtOH when compared to treatment with dopamine alone (Figure 2.5B).

#### Cyclic AMP Analog Stimulation of cAMP Regulated Reporter Gene Expression

Cells expressing DRD1A and AC7 were stimulated with dopamine or 8-bromo-cAMP in the presence or absence of 150 mM ethanol for 3 hours. In cells stimulated with dopamine in the presence of ethanol, a significant increase in cAMP regulated reporter gene expression compared to treatment with dopamine alone was detected. When cells were stimulated with 8-bromo-cAMP, there was no significant change in reporter gene activity with the addition of ethanol



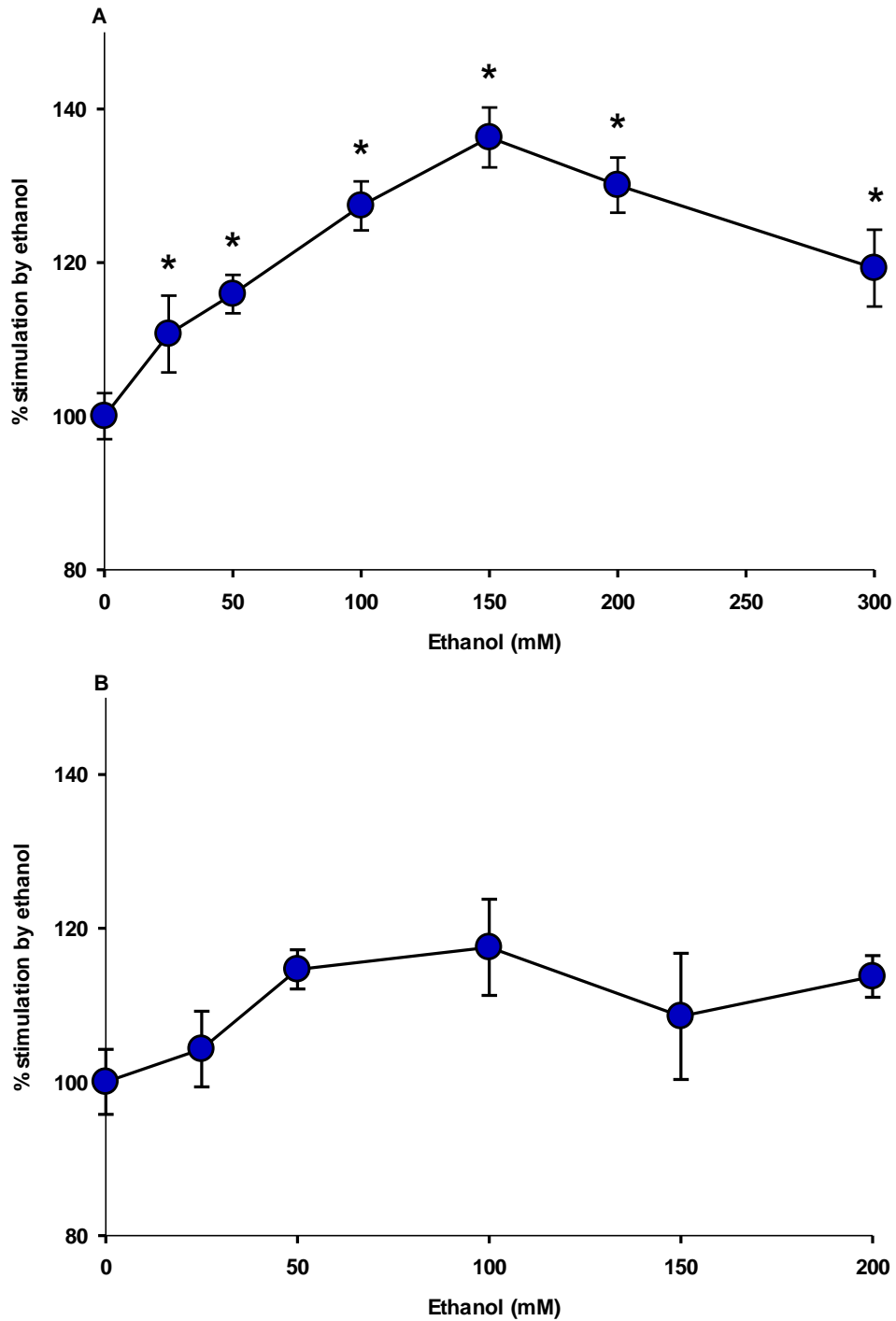


Figure 2.4. Effects of EtOH on cAMP stimulated reporter gene expression in cells expressing DRD1A and AC7 or AC3. (A) Cells expressing AC7 were incubated with 3  $\mu$ M of DA in the presence of EtOH as indicated for 3 hours. Percent stimulation by EtOH is plotted (n = 9 to 12). (B) Treatment of the cells expressing AC3 was similar to that shown in (A) (n = 3). \*Value is significantly greater than basal activity (DA alone) (p < 0.05, 1-way ANOVA).

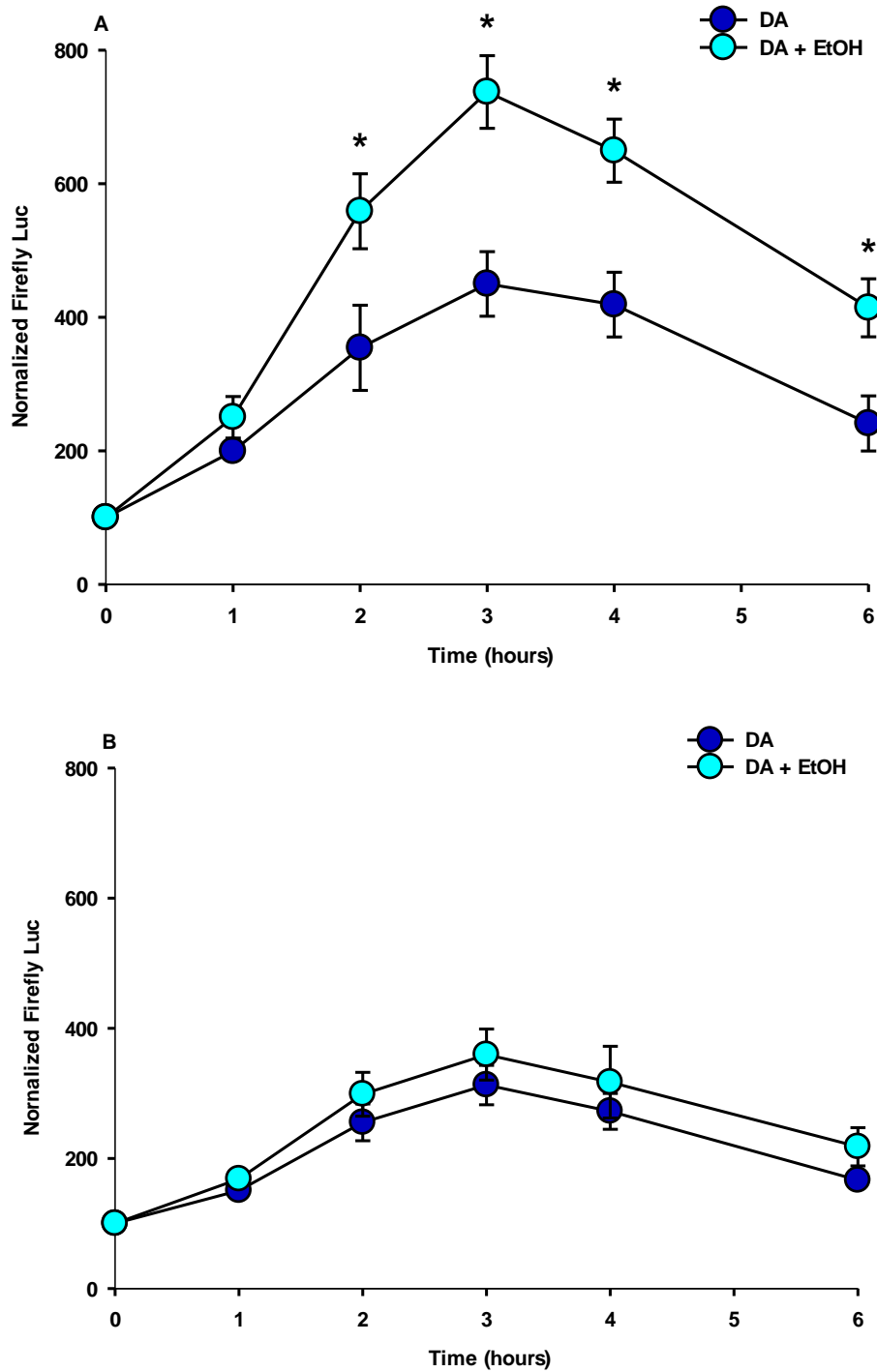


Figure 2.5. Time course of reporter gene activity stimulated by DA and EtOH. (A) Reporter gene activity in cells expressing AC7 and DRD1A. Cells were incubated with 3  $\mu$ M DA in the presence or absence of 150 mM EtOH for 0, 1, 2, 3, 4, 6 hours as indicated (n = 6). (B) Reporter gene activity in cells expressing AC3 and DRD1A (n = 6). Treatment was similar to that shown in (A). \*Value is significantly greater in the presence of EtOH (p < 0.05, 2-way ANOVA).

(Figure 2.6A). In the presence of 8-bromo-cAMP, Firefly luciferase activity increased during the first 3 hours and then gradually decreased. This is similar to the time course observed in the presence of DA. Firefly luciferase activity was not significantly enhanced by ethanol at any time point between 0 and 6 hours (Figure 2.6B).

#### Cyclic AMP Regulated Reporter Gene Expression Stimulated by an Endogenous GPCR

PGE<sub>1</sub> was used to stimulate cAMP generation without an exogenously expressed GPCR. Cells expressing AC7 were stimulated with PGE<sub>1</sub>, ethanol, or PGE<sub>1</sub> in the presence of ethanol for 3 hours. In cells stimulated with PGE<sub>1</sub> in the presence of ethanol, a significant increase in cAMP regulated reporter gene expression compared to basal, PGE<sub>1</sub>, or ethanol alone was detected (Figure 2.7).

#### Effect of Ethanol on CREB Phosphorylation

The level of CREB phosphorylation was examined by western blotting using specific antibodies against CREB and pCREB (Ser133), respectively (Figure 2.8). Cells expressing AC7 were stimulated with PGE<sub>1</sub>, ethanol, or PGE<sub>1</sub> in the presence of ethanol for 30 min; nuclear extracts were prepared after stimulation. The signal intensity of the CREB and pCREB specific bands were normalized using  $\beta$ -actin specific signal as an internal control. No significant changes in the level of CREB or pCREB were detected among the four different incubation conditions. We examined the levels of CREB and pCREB at different time points (5 and 15 min, 1, 2, and 3 hr). However, no differences among the four conditions were detected (data not shown). The experiments were also carried out using cells treated with 0.5 % FBS for 24 hr with similar results (data not shown). When western blotting was performed using cells transfected with a plasmid containing GAL4-CREB, a signal corresponding to this protein was not detected.

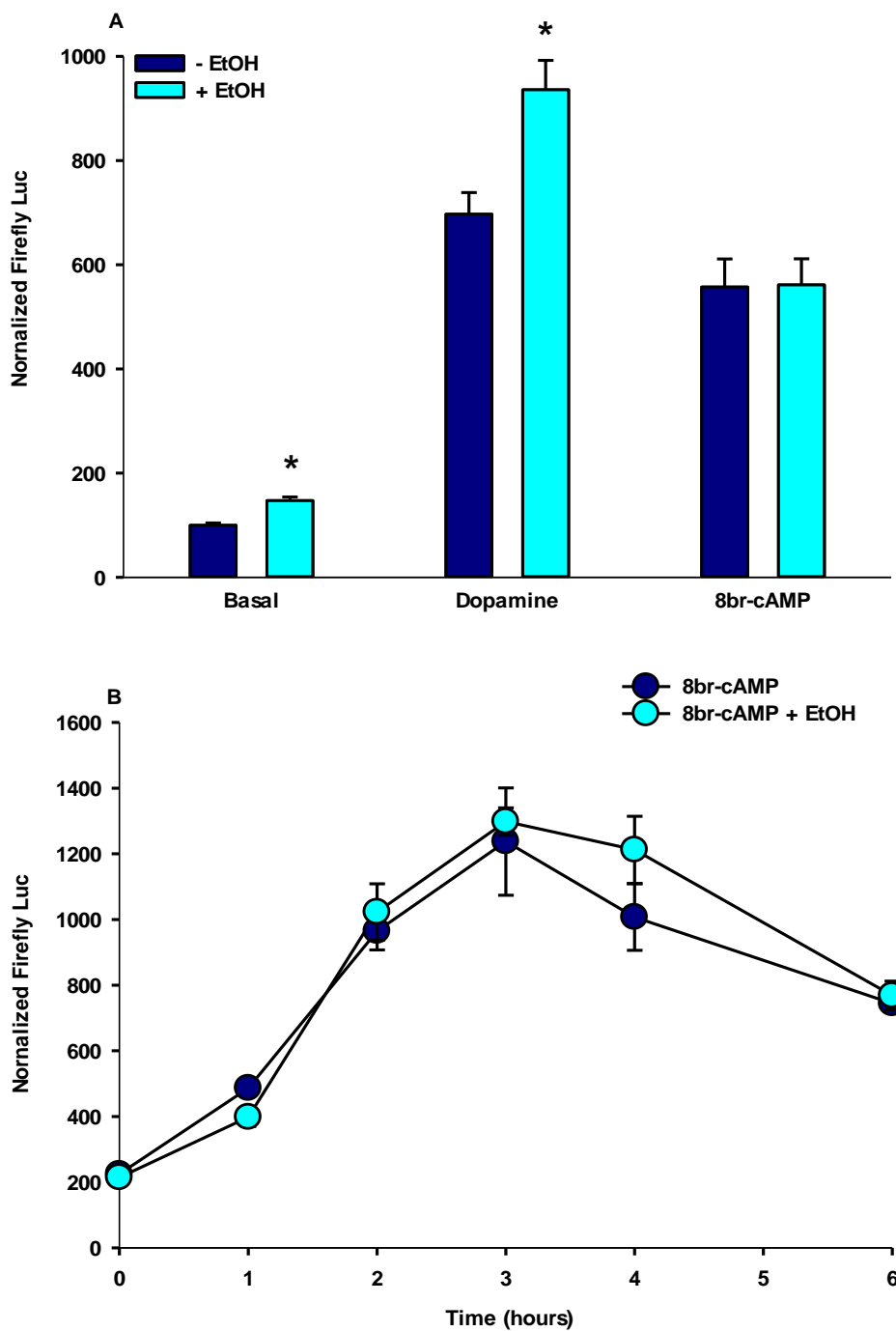


Figure 2.6. Effects of 8Br-cAMP on reporter gene activity in cells expressing DRD1A and AC7. (A) Cells were incubated with 3  $\mu$ M DA or 1 mM 8Br-cAMP in the presence or absence of 150 mM EtOH for 3 hours ( $n = 12$ ). The basal condition received solvent only. \*Value is significantly greater in the presence of EtOH ( $p < 0.05$ , student t-test). (B) Time course of reporter gene activity stimulated by 8Br-cAMP and EtOH. Cells were incubated with 1 mM 8Br-cAMP in the presence or absence of 150 mM EtOH for 0, 1, 2, 3, 4, 6 hours as indicated ( $n = 3$ ) (2-way ANOVA).

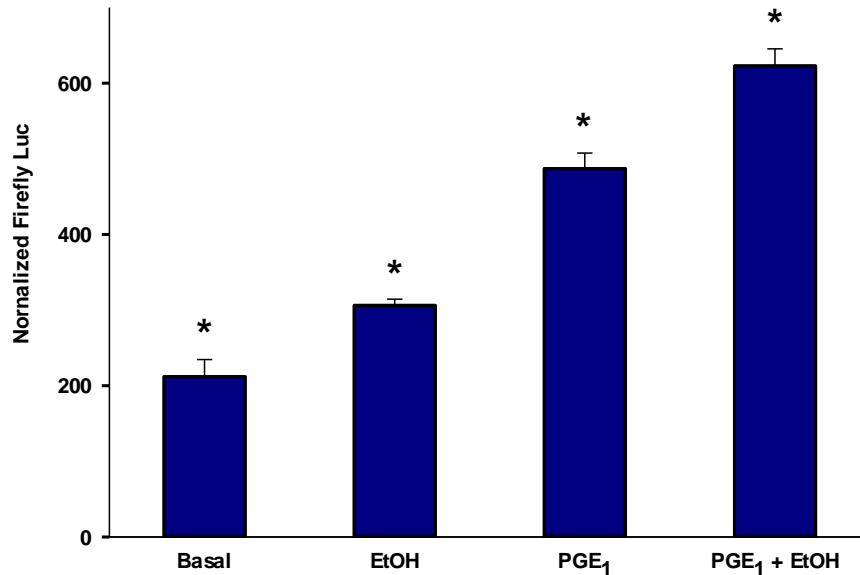


Figure 2.7. Effects of PGE<sub>1</sub> and EtOH on cAMP stimulated reporter gene expression in cells expressing AC7. Cells were incubated with 10  $\mu$ M PGE<sub>1</sub> and 150 mM EtOH as indicated for 3 hours (n = 3). The basal condition received solvent only. \*All pair-wise comparisons are significant (p < 0.05, 1-way ANOVA).

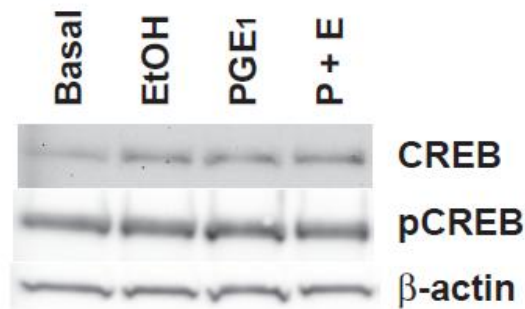


Figure 2.8. Immunodetection of CREB in NIH 3T3 cells. Western blot analysis of nuclear extracts from NIH 3T3 cells incubated in the presence or absence of 10  $\mu$ M PGE<sub>1</sub> and 150 mM EtOH for 30 min. Each lane contained 10  $\mu$ g of protein. Changes in the phosphorylation state of CREB in response to cAMP pathway activation or EtOH treatment could not be detected.

#### Effect of Ethanol on TORC Nuclear Translocation

Western blotting for TORC 1, 2, and 3 was performed using nuclear extracts prepared from cells expressing AC7 stimulated with PGE<sub>1</sub>, EtOH, or PGE<sub>1</sub> + EtOH for 30 min (Figure

2.9A). The signal specific to TORC2 was too weak to be quantified. The intensity of TORC3 in the nuclear extract from cells incubated with PGE<sub>1</sub> and ethanol was higher than the signals obtained from extracts derived from cells incubated with the three other conditions (Figure 2.9B). There was no significant difference detected in TORC1 signals among the four conditions.

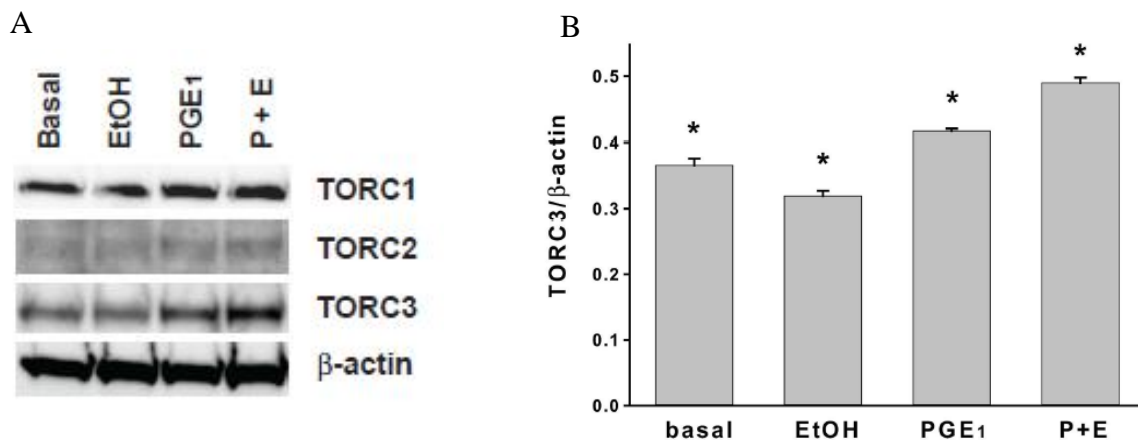


Figure 2.9. Immunodetection of TORCs in NIH 3T3 cells. (A) Western blot analysis of nuclear extracts from NIH 3T3 cells incubated in the presence or absence of 10  $\mu$ M PGE<sub>1</sub> and 150 mM EtOH for 30 min. Each lane contained 10  $\mu$ g of protein. (B) Changes in TORC3 were normalized using  $\beta$ -actin (n = 3). \*All pair-wise comparisons are significant (p < 0.05, 1-way ANOVA).

## Discussion

Previous research suggests that within the cAMP generating system, AC is the target of alcohol's action; alcohol directly interacts with AC molecules to modulate AC activity. These responses occur at physiologically relevant concentrations of ethanol and are AC isoform specific (Yoshimura and Tabakoff, 1995; Yoshimura and Tabakoff, 1999). In this chapter, we hypothesize that the AC isoform expressed in the cells will dictate alcohol's effect on cAMP signaling, which will lead to changes in cAMP inducible gene expression. Cyclic AMP regulated gene expression was evaluated in HEK 293 and NIH 3T3 cell lines. The Firefly luciferase

activity in NIH 3T3 cells responded to cAMP stimulation whereas both Firefly luciferase and Renilla luciferase activity in HEK 293 cells was very low.

Cyclic AMP accumulation assay using a population of NIH 3T3 cells transfected with DRD1A and AC7 or AC3 showed enhancement of cAMP production during stimulation with dopamine. This effect was further enhanced when cells were stimulated with dopamine in the presence of ethanol in AC7 transfected cells. Cells expressing Epac1-camps, DRD1A, and AC7 or AC3 were stimulated with dopamine in the presence or absence of ethanol. In cells expressing AC7, cAMP levels rapidly increased after the addition of dopamine. When ethanol was added together with dopamine, cAMP was further increased. Cyclic AMP levels in cells expressing AC3 followed a similar time course to that of cells expressing AC7 during stimulation with dopamine. However, ethanol had no significant effects on the time course or amount of cAMP in cells expressing AC3. These results confirm our previous observations in Hela and HEK 293 cells (Gupta et al., 2013; Yoshimura and Tabakoff, 1995) and strongly suggest that the isoform specific effect of ethanol on AC activity can be observed in many different types of mammalian cells.

Firefly luciferase activity in cells expressing AC7 and DRD1A showed that dopamine caused a concentration dependent increase in enzymatic activity. Cells expressing AC7 and DRD1A showed that ethanol affects cAMP response in a concentration dependent manner; these effects are also time dependent and AC isoform dependent. Firefly luciferase activity is significantly increased by ethanol at 25 and 50 mM in AC7 expressing cells. Regardless of the ethanol concentration used to stimulate the cells, enhancement was not evident in AC3 expressing cells. The effect of ethanol on Firefly luciferase activity of cells expressing AC7 reaches its maximum at 150 mM and decreases at 200 mM. This result differs from the ethanol

response of cAMP accumulation in HEK293 cells expressing AC7 previously observed (Yoshimura and Tabakoff, 1999). Although this decrease does not have physiological relevance, it may assist in elucidating the mechanism by which ethanol affects the function of AC and other proteins involved in cAMP regulated gene expression.

To further examine the effect of alcohol on cAMP regulated reporter gene expression, we utilized 8-bromo-cAMP to uncouple AC from the cAMP signaling pathway. 8-bromo-cAMP increased the cAMP driven reporter gene expression in NIH 3T3 cells in a time- and concentration-dependent manner. 8-bromo-cAMP induced reporter gene expression was not significantly affected by ethanol, further indicating that the alcohol effect on cAMP regulated reporter gene expression is due to the enhancement in the AC activity caused by ethanol; there is no significant ethanol effect occurring downstream of cAMP generation.

In cells transfected with AC7, cAMP regulated reporter gene expression was stimulated with PGE<sub>1</sub>; this activity was further enhanced by ethanol. These results are similar to those observed in cells stimulated with dopamine, indicating that enhancement of cAMP regulated reporter gene expression by ethanol can be observed by stimulating endogenous GPCRs. The overall results of this study show that cAMP regulated reporter gene expression is enhanced by ethanol in cells expressing AC7.

Several previous studies with NIH 3T3 cells have shown that the level of pCREB increased when cAMP signaling was stimulated (Chen et al., 2002; Johannessen et al., 2004b; Mayr and Montminy, 2001). In addition, incubation of cells with ethanol increased the level of pCREB in NG108–15 cells (Constantinescu et al., 1999) and NIH 3T3 cells (Roman et al., 2005). However, in the current study the level of pCREB in nuclear extracts from NIH 3T3 cells was not significantly increased by stimulation with PGE<sub>1</sub>, ethanol, or PGE<sub>1</sub> with ethanol. Levels



of CREB and pCREB were also examined at varying time points; no differences among the four conditions were detected (data not shown). Changes in the phosphorylation state of CREB in response to cAMP pathway activation or ethanol treatment were not detectable. The notable difference between the current study and previous studies is that the basal level of Ser133 phosphorylation of CREB in the NIH 3T3 in our laboratory is much higher than previously reported. Phosphorylation of Ser133 of CREB can be achieved by many protein kinases including PKA (Johannessen et al., 2004a). Culture conditions in the current study may have caused a high basal level of pCREB or our NIH 3T3 cells may have unknown alterations compared to cells in previous studies. In either case, the changes in pCREB level did not correlate with the changes in expression level of the cAMP regulated reporter gene.

We then examined the levels of TORC1-3 in the nuclear extracts of NIH 3T3 cells. TORCs translocate from the cytoplasm to the nucleus by stimulation of cAMP signaling and participate in the activation of CRE-driven transcription (Bittinger et al., 2004). Western blotting of TORC1-3 detected signals of expected size, however, TORC2 signals were too weak to quantify. TORC1 signals did not change with different stimulation conditions. TORC3 signals showed that PGE<sub>1</sub> increases TORC3 translocation to the nucleus; ethanol further enhances this translocation. The difference in response of TORC1 and TORC3 to the stimulation conditions could be due to several reasons. Lack of nuclear translocation could be due to a difference in sensitivity of TORC1 and TORC3 to the translocation signal, or there could be a difference in sub-cellular localization of TORC1 and TORC3 and their accessibility to the translocation signal. Alternatively, this could be due to different populations of cells within our NIH 3T3 cells which differentially express TORC1 and TORC3, thus affecting the translocation signaling

cascade. Among the protein factors examined, changes of TORC3 appear to correlate best with the observed changes in the expression level of the cAMP regulated reporter gene.

In conclusion, our findings indicate that the ethanol effect on cAMP regulated reporter gene expression is due to a change in the amount of cAMP present in the cells, which is dictated by the AC isoform expressed in the cells. The alcohol effect observed on AC is time- and concentration-dependent. These results show that ethanol's effect on AC activity plays a major role on the expression of a cAMP regulated reporter gene. The increase in cAMP by addition of ethanol in cells expressing AC7 is correlated with the increase in reporter gene activity and the level of TORC3 in the nucleus. Further research is needed to elucidate the role of specific AC isoform in the effect of ethanol on cAMP regulated gene expression *in vivo*.

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**CHAPTER 3.**  
**ROLE OF AC7 IN ALCOHOL'S EFFECT ON CYCLIC AMP REGULATED**  
**REPORTER GENE EXPRESSION IN RAW 264.7 AND BV-2 IMMUNE CELLS**

**Introduction**

Cyclic AMP is a ubiquitous second messenger molecule that regulates many diverse biological processes including lipid and sugar metabolism, as well as cell growth, cell differentiation, learning, and memory. Previous research indicates that each of the nine membrane-bound AC isoforms has a distinct tissue distribution and the activity of each isoform is uniquely regulated by a variety of factors including  $\beta\gamma$  subunits of G proteins, calmodulin, calcium, protein kinase A (PKA), and protein kinase C (Cooper, 2003; Sadana and Dessauer, 2009; Sunahara and Taussig, 2002).

It is well established that cAMP regulates immune responses. Cyclic AMP is known for its immunosuppressive effects but is also required for proper development of the immune system (Boer et al., 2003; Houslay et al., 2005; Sanders, 2012). Some proteins in the cAMP signaling pathway have been proposed as targets for inflammatory disease therapies. Adrenergic receptor agonists suppress the proinflammatory activities of T lymphocytes, mast cells, monocytes/macrophages, basophils, eosinophils, and neutrophils via cAMP signaling (Theron et al., 2013). Research also suggests that alcohol induces changes in inflammatory and immune responses. Alcohol use increases the incidence of pneumonias and other infections and can negatively affect recovery time. Experimental and clinical evidence indicates that alcohol use can cause various abnormalities in the immune system (Szabo and Mandrekar, 2009).

Ethanol alters the cAMP signaling pathways in the brain and various tissues in animal models as well as model cell culture systems. Acute exposure to ethanol enhances receptor-stimulated and/or stimulatory G protein (Gs)-activated AC activity, while chronic exposure to

ethanol often decreases AC activity (Tabakoff and Hoffman, 1998). ACs play an important role in cAMP-dependent activation of PKA as well as regulating cAMP response element binding protein (CREB) function via its phosphorylation. CREB is a potential ethanol target and plays a central role in the process of alcohol addiction (Pandey et al., 2003; Pandey et al., 2004). Phosphorylated CREB inhibits NF- $\kappa$ B activation by blocking the binding of CREB binding protein (CBP/p300) to the NF- $\kappa$ B complex, limiting proinflammatory responses (Wen et al., 2010). CREB also induces anti-apoptotic survival signals in monocytes and macrophages, enhancing host immune responses (Park et al., 2005). Recently, a new family of tissue specific and cell specific CREB co-activators known as transducer of regulated CREB activity (TORC) that bind to the bZip domain of CREB have been discovered (Conkright et al., 2003; Iourgenko et al., 2003). TORC1-3 activate CRE-driven transcription. The TORC pathway may play a role in immune function through regulation of cAMP regulated gene expression.

The role of cAMP in the effect of drinking alcohol on immune function has not been well documented. Studies have demonstrated that AC7 is highly expressed in immune cells and plays an important role in the immune response (Duan et al., 2010; Jiang et al., 2013). AC7 is also the most ethanol responsive AC isoform (Yoshimura and Tabakoff, 1995). We hypothesize that alcohol modulates cAMP signaling in immune cells by enhancing the activity of AC7; therefore, AC7 may play a role in ethanol's effects on immune function. To examine this hypothesis, we utilized well-established, AC7 expressing mammalian cell lines. RAW 264.7 cells are a mouse derived macrophage cell line that has been well characterized in regards to its cellular signaling network. Many receptor-specific ligands have been studied, alone as well as in pair-wise combinations for: cAMP synthesis, Ca<sup>2+</sup> mobilization, cytokine production, and phosphorylation of many signaling proteins which have provided critical knowledge regarding cellular responses

to many extracellular stimuli (Jiang et al., 2007; Jiang et al., 2008; Natarajan et al., 2006). BV-2 cells are a mouse derived microglia cell line. Analysis of mRNA expression of various signaling proteins in BV-2 cells has shown that AC7 is the dominant AC isoform expressed in this cell line (Atwood et al., 2011). Fluorescent resonance energy transfer (FRET) based cAMP sensor, Epac1-camp, and radioisotope based cAMP accumulation assays were used to monitor cAMP levels within the cells. Cyclic AMP regulated gene expression was examined using a CRE-Luc reporter gene. The changes in TORC nuclear translocation and the phosphorylation of CREB were measured via western blotting. The results provide insight into the molecular mechanisms of the alcohol effect on cAMP inducible gene expression in immune cells, which predominantly express the AC7 isoform.

## **Materials and Methods**

### Reagents

Ethanol (EtOH) was purchased from Sigma-Aldrich (St. Louis, MO). Prostaglandin E<sub>1</sub> (PGE<sub>1</sub>), sphingosine-1-phosphate (S1P), and thrombin were purchased from Cayman Chemical (Ann Arbor, MI). [ $\alpha$ -<sup>32</sup>P]ATP was purchased from Perkin Elmer (Boston, MA). [2,8-<sup>3</sup>H]adenine and [8-<sup>14</sup>C]cAMP were purchased from Moravek Biochemicals (Brea, CA).

### Plasmid DNA

Plasmids containing the coding sequences for Epac1-cAMP (Nikolaev and Lohse, 2006), pCRE-Luc (Agilent, Santa Clara, CA), SV40-Renilla reporter plasmid pRL-SV40 (Promega, Madison, WI), and an expression plasmid for green fluorescent protein (GFP) (Clontech, Mountain View, CA) were used.



### Cell Culture and Transfection

RAW 264.7 cells were obtained from ATCC (Manassas, VA). BV-2 cells were obtained from Banca Biologica e Cell Factory (Genova, Italy). RAW 264.7 cells and BV-2 cells were maintained separately in 30 ml Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum, penicillin (50ug/ml), streptomycin (50ug/ml), and neomycin (100ug/ml) in plastic flasks (225cm<sup>2</sup>) at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. Cells were transiently transfected using Calfectin (SignaGen, Rockville, MD) according to the manufacturer's instructions. Transfection efficiency was monitored by observing the expression of green fluorescent protein (GFP) or Epac1-cAMP using an epifluorescence microscope.

### Cyclic AMP Accumulation Assay

The amount of cAMP in the transfected BV-2 cells was assessed by the cAMP accumulation assay as described previously (Hasanuzzaman and Yoshimura, 2010). Briefly, the intracellular ATP pool was labeled with 3.0 µCi/ml of [2,8-<sup>3</sup>H]adenine. To determine the effect of ethanol, cells were treated with varying ethanol concentrations together with 1 µM PGE<sub>1</sub> for 1 minute at 37°C. The reaction was terminated by adding 50 µl of 100% (w/v) trichloroacetic acid. ATP and cAMP contents of each well were separated through Dowex 50 and neutral alumina columns as described previously (Salomon et al., 1974) and quantified by liquid scintillation spectrometer. [ $\alpha$ -<sup>32</sup>P]ATP and [8-<sup>14</sup>C]cAMP were added as internal standards in order to monitor recovery of ATP and cAMP through column chromatography.

### Live Cell Fluorescence Imaging

A 15mm glass coverslip was coated with Poly(ethyleneimine) solution (PEI) (Sigma Aldrich, St. Louis, MO) for 30 minutes. Transfected RAW 264.7 cells or BV-2 cells were added to the coverslip 24 hours prior to the experiment. The coverslip was assembled as described in

Chapter 2. The fluorescent imaging workstation, filter sets, and buffer composition were described previously (Gupta et al., 2013). Drugs were added via perfusion for the durations indicated in the figures. Three fluorescent images at each time point were captured using filters set specifically for CFP, YFP, and FRET, respectively. Images were captured every 3 sec for the duration of time-lapse experiments.

### FRET Measurements

The method of sensitized FRET measurement was previously described (Sorkin et al., 2000). Background images were subtracted from the raw images prior to FRET calculations. Corrected FRET (cFRET) and normalized FRET (nFRET) were calculated as previously described (Gupta et al., 2013). nFRET values were normalized by dividing by the mean nFRET value of the first 18 time points.

### Luciferase Assay

24 hours post-transfection, cells were transferred to 24-well culture plates. RAW 264.7 cells were treated with PGE<sub>1</sub> or 8-bromo-cAMP in the presence or absence of ethanol. Cells were lysed and assayed for Firefly and Renilla luciferase activities using a Dual Luciferase Assay System (Promega, Madison, WI) and a Synergy HT plate reader (BioTek Instruments, Winooski, VT) according to the manufacturer's instructions. Firefly luciferase activities were normalized against Renilla luciferase activities.

### Nuclear Extract Preparation and Western Blotting

Following stimulation, RAW 264.7 cells and BV2 cells were washed with phosphate-buffered saline (PBS) and collected via scraping. Nuclear extracts were prepared using a NE-PER kit from Pierce (Rockford, IL). Protein concentration in the extracts was determined using the BCA protein assay kit (Pierce, Rockford, IL). The extracts were stored at -80°C prior to use.

Proteins (20  $\mu$ g per lane) were separated by 12.5% SDS-PAGE and transferred to a nitrocellulose membrane. Western blotting was carried out using following primary antibodies according to manufacturer protocol: anti-CREB (Millipore, Billerica, MA), anti-phospho-CREB (pCREB) (Millipore, Billerica, MA), TORC1 (GeneTex, Irvine, CA), TORC2 (Abcam, Cambridge, MA), TORC3 (GeneTex, Irvine, CA), and  $\beta$ -actin (Cell Signaling, Danvers, MA). Appropriate secondary antibodies conjugated with horseradish peroxidase were obtained from Jackson ImmunoResearch (West Grove, PA). Signals were visualized by chemiluminescence using ECL detection reagents (GE Healthcare, Piscataway, NJ), images were captured using a ChemiDoc Touch imaging system (Bio-Rad, Hercules, CA), quantified using ImageJ software (Schneider et al., 2012), and normalized to  $\beta$ -actin for comparison.

### Statistics

Values are expressed as mean  $\pm$  standard error of mean (SEM). Student's t-test, one-way ANOVA, two-way ANOVA, or two-way repeated-measures ANOVA were used to evaluate differences in values as indicated in figure legends. After ANOVA, pairwise comparisons were carried out by Holm–Sidak method. Analyses were carried out using SigmaStat (SyStat Software, San Jose, CA).

## **Results**

### Effect of Ethanol on cAMP in RAW 264.7 and BV-2 Cells

RAW 264.7 cells expressing Epac1-cAMP were stimulated with PGE<sub>1</sub> in the presence or absence of ethanol for 2 minutes to monitor real-time changes in cAMP. Normalized nFRET values were plotted over time as an indicator of cAMP (Figure 3.1). nFRET rapidly decreased after the addition of PGE<sub>1</sub>; this decrease indicates an increase in cAMP. The value of nFRET reached its minimum 60 seconds after addition of PGE<sub>1</sub> and remained constant during the

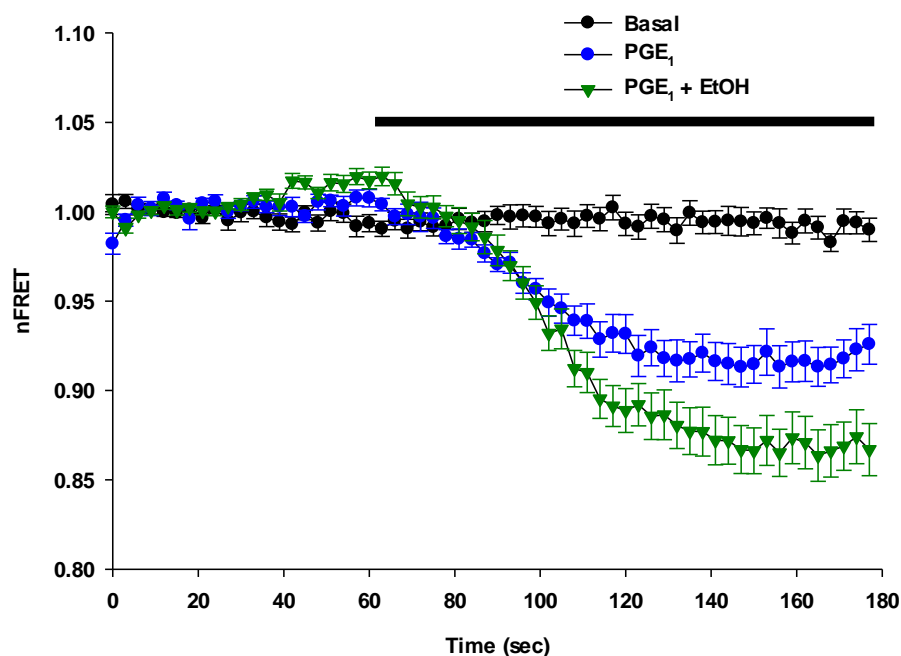


Figure 3.1. Effects of PGE<sub>1</sub> and EtOH exposure on cAMP in RAW 264.7 cells. Data presented as mean  $\pm$  SEM (Basal, n = 16; PGE<sub>1</sub>, n = 14; PGE<sub>1</sub> + EtOH, n = 20). nFRET values are plotted over time. The bar on the top of the graph indicates 2 minute duration of PGE<sub>1</sub> (10 $\mu$ M) and PGE<sub>1</sub> + EtOH (200 mM) treatment. Values past 100 seconds are significantly smaller in the presence of PGE<sub>1</sub> + EtOH compared to basal or PGE<sub>1</sub> alone (p < 0.05, 2-way repeated-measures ANOVA).

remainder of the PGE<sub>1</sub> treatment (~60 seconds). When ethanol was added with PGE<sub>1</sub>, nFRET was further decreased. nFRET reached its minimum ~70 seconds after addition of PGE<sub>1</sub> and ethanol.

In order to establish that AC7 is the main AC isoform expressed in BV-2 cells, the AC7 specific activators thrombin and sphingosine-1-phosphate were utilized. The cAMP accumulation assay using a population of BV-2 cells clearly showed enhancement of cAMP production during the 1-minute stimulation with PGE<sub>1</sub>. Cyclic AMP accumulation was further enhanced by PGE<sub>1</sub> in the presence of ethanol as well as the AC7 specific activators (Figure 3.2). Incubation without PGE<sub>1</sub> or the addition of S1P, thrombin, or ethanol alone did not cause any

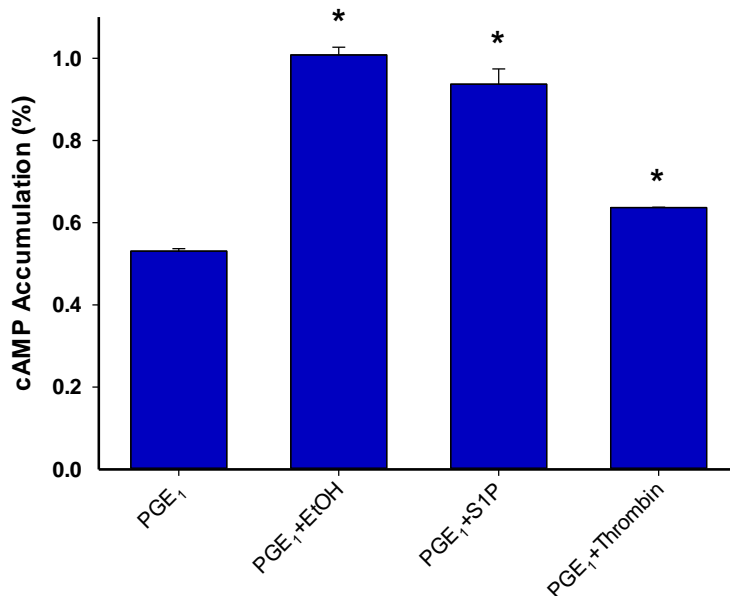


Figure 3.2. Effects of PGE<sub>1</sub>, EtOH, S1P, and thrombin on cAMP accumulation in BV-2 cells. Assays were carried out in the presence of PGE<sub>1</sub> (1 μM) ± EtOH (200 mM), S1P (100 nM), or thrombin (1 unit/ml). Percent increase in cAMP accumulation are displayed as mean ± SEM (n = 3). \*Values are significantly greater than PGE<sub>1</sub> alone (p < 0.05, 1-way ANOVA).

changes in cAMP accumulation (data not shown). Cyclic AMP in BV-2 cells was stimulated with either sphingosine-1-phosphate or thrombin. BV-2 cells stimulated with PGE<sub>1</sub> and each of the AC7 specific activators followed a similar time course to that RAW 264.7 cells stimulated with PGE<sub>1</sub> and ethanol (Figure 3.3). The increase in the amount of cAMP present in the cells in response to stimulation with PGE<sub>1</sub> and AC7 specific activators indicate that AC7 is the main AC isoform expressed in BV-2 cells. The effect of ethanol on BV-2 cells are similar to our previous observations in Hela cells, HEK 293 cells, and NIH 3T3 cells expressing AC7, further indicating that AC7 is the dominate isoform present in BV-2 cells (Gupta et al., 2013; Hill et al., 2016; Yoshimura and Tabakoff, 1995). During stimulation with PGE<sub>1</sub> in the presence or absence of ethanol, cAMP levels of BV-2 cells followed a similar time course to RAW 264.7 cells (Figure 3.4). These results further suggest that the effect of ethanol on AC7 activity can be observed in

many different mammalian cell types. Cyclic AMP accumulation in BV-2 cells showed that ethanol caused a concentration dependent enhancement of cAMP production in the presence of PGE<sub>1</sub> (Figure 3.5). The results are reminiscent of previous observations in which AC7 transfected NIH 3T3 cells and human embryonic kidney (HEK) 293 cells were utilized (Hill et al., 2016; Yoshimura et al., 2006).

#### Effect of PGE<sub>1</sub> and Ethanol on cAMP Regulated Reporter Gene Expression in RAW 264.7 Cells

Reporter gene (Firefly luciferase) activity showed significant enhancement during a 3 hour incubation period in the presence of varying concentrations of PGE<sub>1</sub> (Figure 3.6). PGE<sub>1</sub> gave rise to a concentration dependent increase in Firefly luciferase activity, reaching maximum activity between 3 and 30 μM PGE<sub>1</sub>. RAW 264.7 cells were stimulated with PGE<sub>1</sub> and varying concentrations of ethanol for 3 hours. A significant increase in cAMP regulated gene expression compared to PGE<sub>1</sub> alone was detected at the lowest ethanol concentration (25 mM) (Figure 3.7).

Firefly luciferase activity reached its maximum at 125 mM ethanol. The results indicate that the effect of ethanol on cAMP regulated gene expression in RAW 264.7 cells is ethanol concentration-dependent and is similar to the ethanol effect on cAMP regulated reporter gene expression observed in NIH 3T3 cells expressing AC7 (Hill et al., 2016). RAW 264.7 cells were stimulated with PGE<sub>1</sub> in the presence or absence of ethanol at varying time points. Significant increase in cAMP regulated reporter gene expression was detected at all time points past 2 hours by the addition of PGE<sub>1</sub> and ethanol when compared to PGE<sub>1</sub> alone (Figure 3.8). The results indicate that the effect of ethanol on cAMP regulated gene expression is time-dependent. 8-bromo-cAMP was utilized to uncouple AC from the cAMP signaling pathway. RAW 264.7 cells were stimulated with PGE<sub>1</sub> or 8-bromo-cAMP in the presence or absence of ethanol for 3 hours. In cells stimulated with PGE<sub>1</sub> and ethanol, a significant increase in cAMP regulated reporter

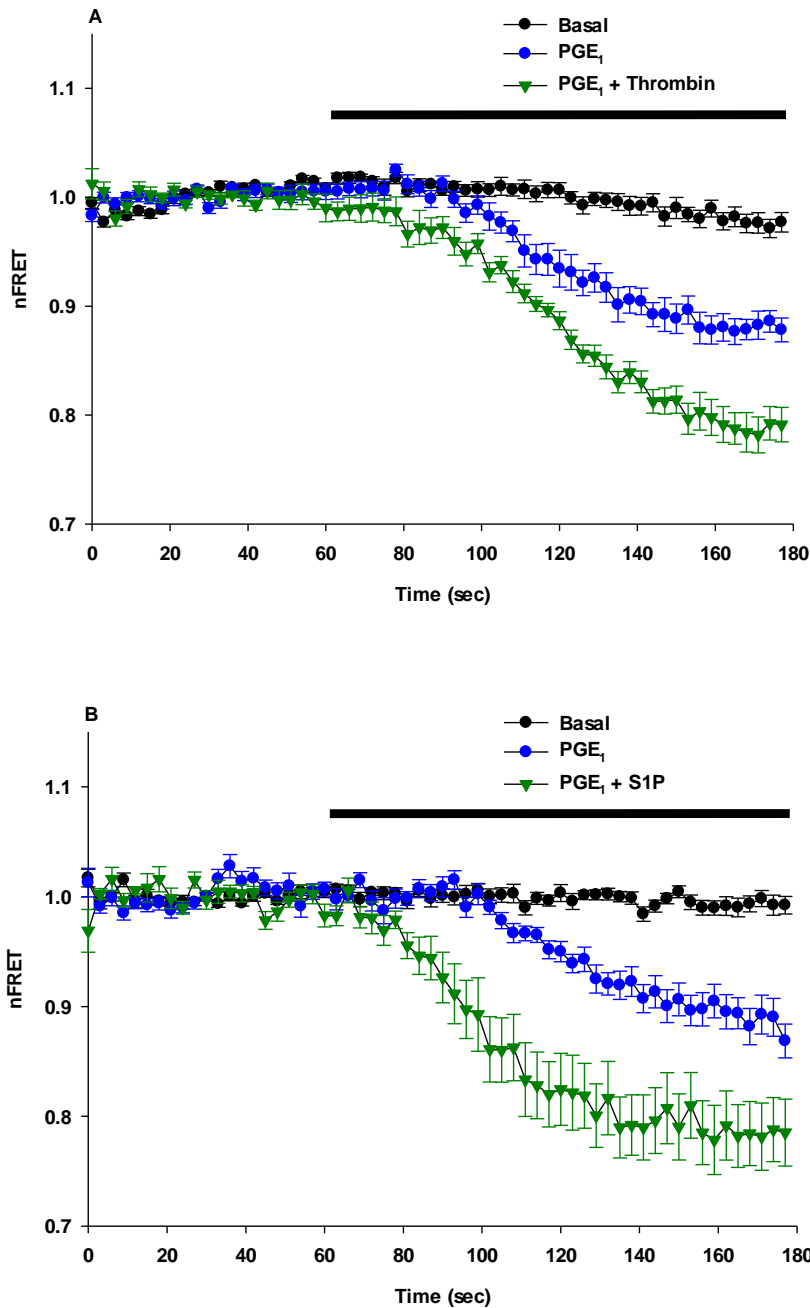


Figure 3.3. Effects of PGE<sub>1</sub>, thrombin, and S1P exposure on cAMP in BV-2 cells. (A) Real-time monitoring of PGE<sub>1</sub> (10 $\mu$ M) and thrombin (1 unit/ml) exposure on cAMP (Basal, n = 12; PGE<sub>1</sub>, n = 12; PGE<sub>1</sub> + thrombin, n = 9). nFRET values are plotted over time. The black bar indicates duration of treatment. Data presented as mean  $\pm$  SEM; values past 85 seconds are significantly smaller in the presence of thrombin + EtOH than basal or thrombin alone ( $p < 0.05$ , 2-way repeated-measures ANOVA). (B) Real-time monitoring of PGE<sub>1</sub> and S1P (100  $\mu$ M) exposure on cAMP (Basal, n = 15; PGE<sub>1</sub>, n = 11; PGE<sub>1</sub> + S1P, n = 8). Duration of treatment and statistical analysis were similar to (A). Values past 100 seconds are significantly smaller in the presence of S1P + EtOH than basal or S1P alone.

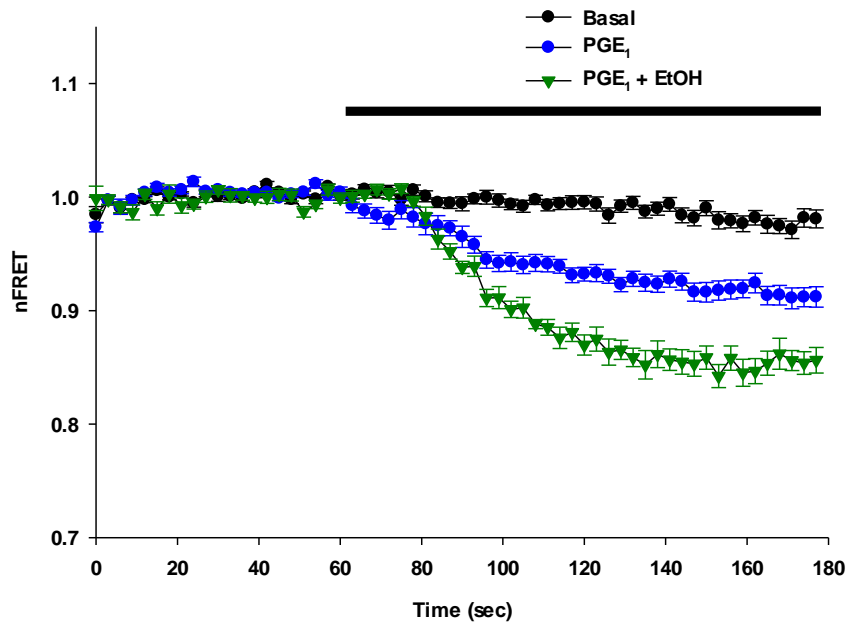


Figure 3.4. Effects of PGE<sub>1</sub> and EtOH exposure on cAMP in BV-2 cells. Data presented as mean  $\pm$  SEM (Basal, n = 18; PGE<sub>1</sub>, n = 22; PGE<sub>1</sub> + EtOH, n = 12). nFRET values are plotted over time. The black bar indicates duration of PGE<sub>1</sub> (10 $\mu$ M) and PGE<sub>1</sub> + EtOH (200 mM) treatment. Values past 85 seconds are significantly smaller in the presence of PGE<sub>1</sub> + EtOH than basal or PGE<sub>1</sub> alone ( $p < 0.05$ , 2-way repeated-measures ANOVA).

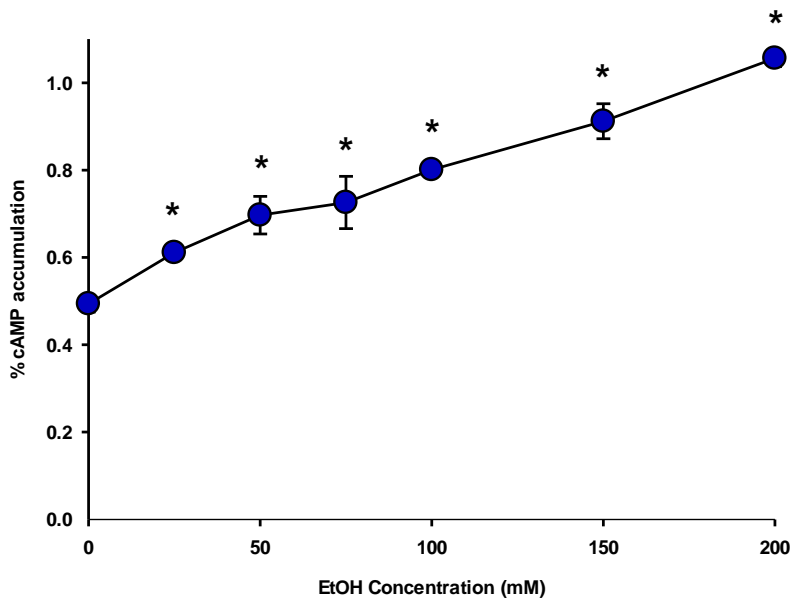


Figure 3.5. Effects of EtOH on cAMP accumulation in BV-2 cells. Assays were carried out with PGE<sub>1</sub> (1  $\mu$ M) in the presence of 0, 25, 50, 75, 100, 150, 200 mM EtOH. Cyclic AMP accumulation is presented as mean  $\pm$  SEM (n = 3). \*Value is significantly greater than PGE<sub>1</sub> alone (0 mM EtOH) ( $p < 0.05$ , 1-way ANOVA).



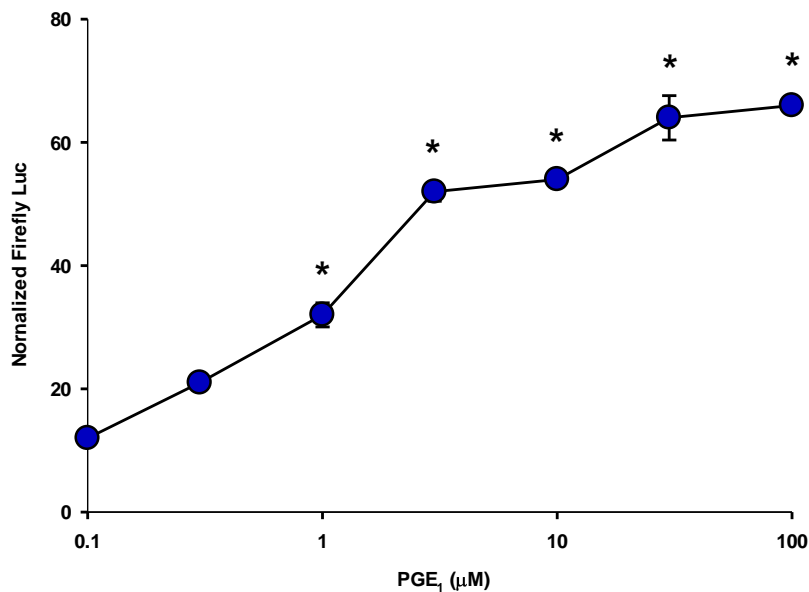


Figure 3.6. Effects of PGE<sub>1</sub> on cAMP stimulated reporter gene expression in RAW 264.7 cells. Cells were incubated in the presence of 0, 0.1, 0.3, 1, 3, 10, and 30 μM PGE<sub>1</sub> for 3 hours. Basal activity in the absence of PGE<sub>1</sub> was 9.0 ± 0.32. Data are shown as mean ± SEM (n = 3). \*Value is significantly greater than basal activity (p < 0.05, 1-way ANOVA).

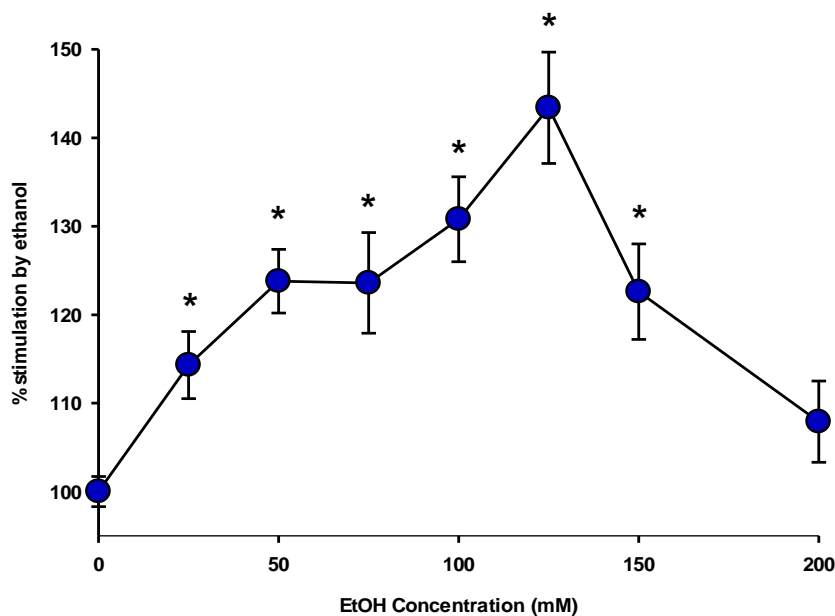


Figure 3.7. Effects of EtOH on cAMP stimulated reporter gene expression in RAW 264.7 cells. Cells were incubated with 1 μM PGE<sub>1</sub> in the presence of 0, 25, 50, 75, 100, 125, 150, 200 mM EtOH for 3 hours. Percent stimulation by EtOH is plotted (n = 9 to 18). \*Value is significantly greater than PGE<sub>1</sub> alone (p < 0.05, 1-way ANOVA).

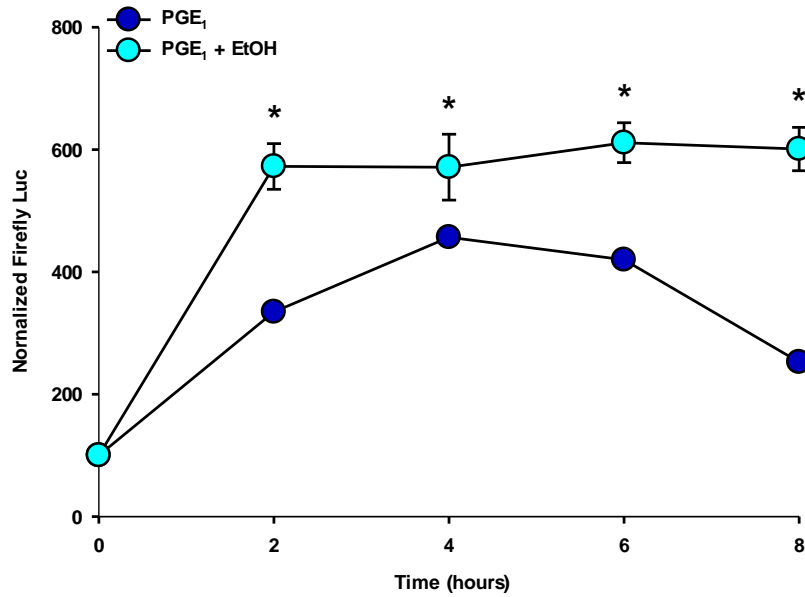


Figure 3.8. Time course of reporter gene activity stimulated by PGE<sub>1</sub> and EtOH in RAW 264.7 cells. Cells were incubated with 1  $\mu$ M PGE<sub>1</sub> in the presence or absence of 150 mM EtOH for 0, 1, 2, 3, 4, 6 hours as indicated (n = 3). \*Value is significantly higher than PGE<sub>1</sub> alone (p < 0.05, 1-way ANOVA).

gene expression compared to PGE<sub>1</sub> alone was detected. When cells were stimulated with 8-bromo-cAMP, there was no significant change in reporter gene activity with the addition of ethanol (Figure 3.9). These results indicate that the effect of ethanol on cAMP regulated reporter gene expression is due to the enhancement of AC7 activity by ethanol. Firefly luciferase and Renilla luciferase activity in BV-2 cells was too weak to quantify (data not shown).

#### Effect of Ethanol on CREB Phosphorylation in RAW 264.7 and BV-2 Cells

The level of CREB phosphorylation in nuclear extracts from RAW 264.7 cells and BV-2 cells was examined by western blotting using specific antibodies against CREB and pCREB (Ser133). Cells were stimulated with medium alone (basal), PGE<sub>1</sub>, ethanol, or PGE<sub>1</sub> and ethanol for 30 minutes; nuclear extracts were prepared after stimulation. The signal intensity of the CREB and pCREB specific bands were normalized using  $\beta$ -actin specific signal as an internal control. No significant changes in the level of CREB or pCREB were detected among the four

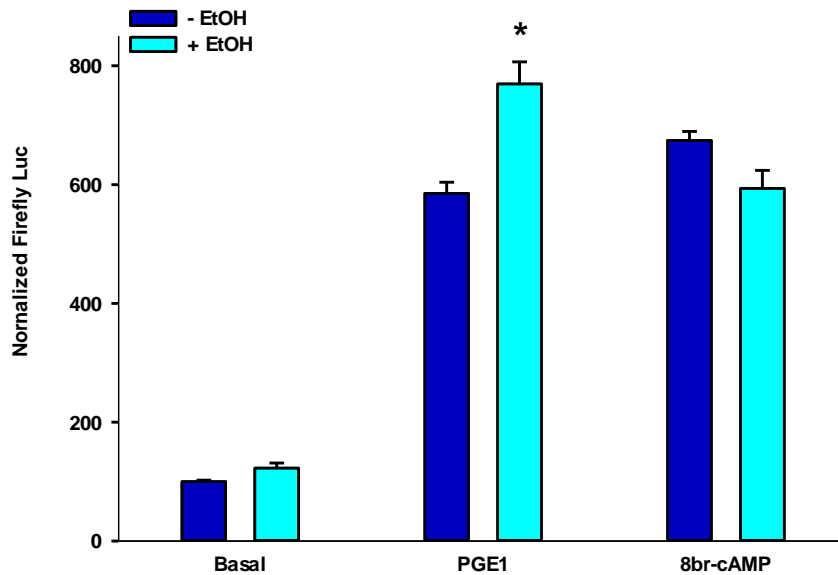


Figure 3.9. Effects of 8-bromo-cAMP on reporter gene expression in RAW 264.7 cells. Cells were incubated with 1  $\mu$ M PGE<sub>1</sub> or 0.25 mM 8-bromo-cAMP  $\pm$  100 mM EtOH for 3 hours (n = 3). The basal condition received solvent only. \*Value is significantly higher in the presence of EtOH (p < 0.05, student t-test).

different incubation conditions in RAW 264.7 cells or BV-2 cells (Figure 3.10). The levels of CREB and pCREB were examined at different time points (5 and 15 min, 1, 2, and 3 hr) in RAW 264.7 cells. However, no differences among the four time points were detected (data not shown). The experiments were also carried out using cells treated with 0.5% FBS for 24 hr with similar results (data not shown).

#### Effect of ethanol on TORC nuclear translocation in RAW 264.7 and BV-2 cells

Western blotting for TORC1-3 was performed using nuclear extracts prepared from RAW 264.7 and BV-2 cells stimulated with PGE<sub>1</sub>, ethanol, or PGE<sub>1</sub> and ethanol for 30 minutes. The signal specific to TORC2 and TORC3 showed an enhancement of nuclear translocation in RAW 264.7 cells treated with PGE<sub>1</sub> and ethanol (Figure 3.11). Signal intensity of TORC1 was similar to that of TORC2 and TORC3, however, there was no significant difference detected in TORC1 signals among the four conditions in RAW 264.7 cells (data not shown). The signal

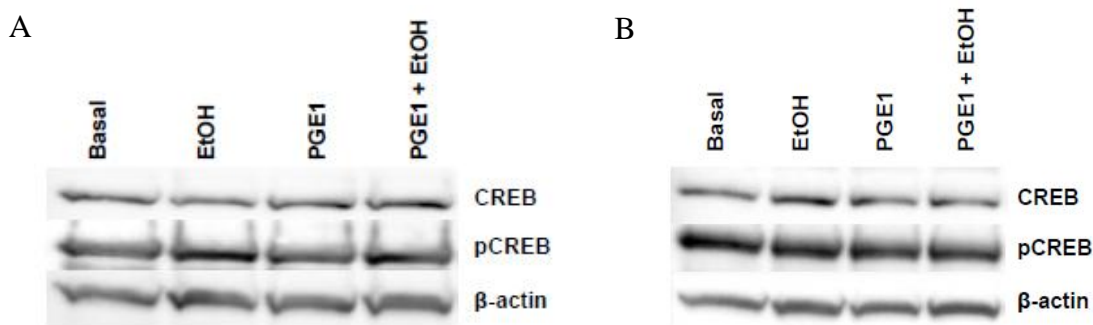


Figure 3.10. Immunodetection of CREB and pCREB in RAW 264.7 and BV-2 cells. (A) RAW 264.7 cells were incubated with medium alone (basal), PGE<sub>1</sub> (10 μM), EtOH (150 mM), or PGE<sub>1</sub> +EtOH for 30 min. Each lane contained 20 μg of protein. (B) Western blot analysis of nuclear extracts from BV-2 cells. Cell treatment was similar to (A).

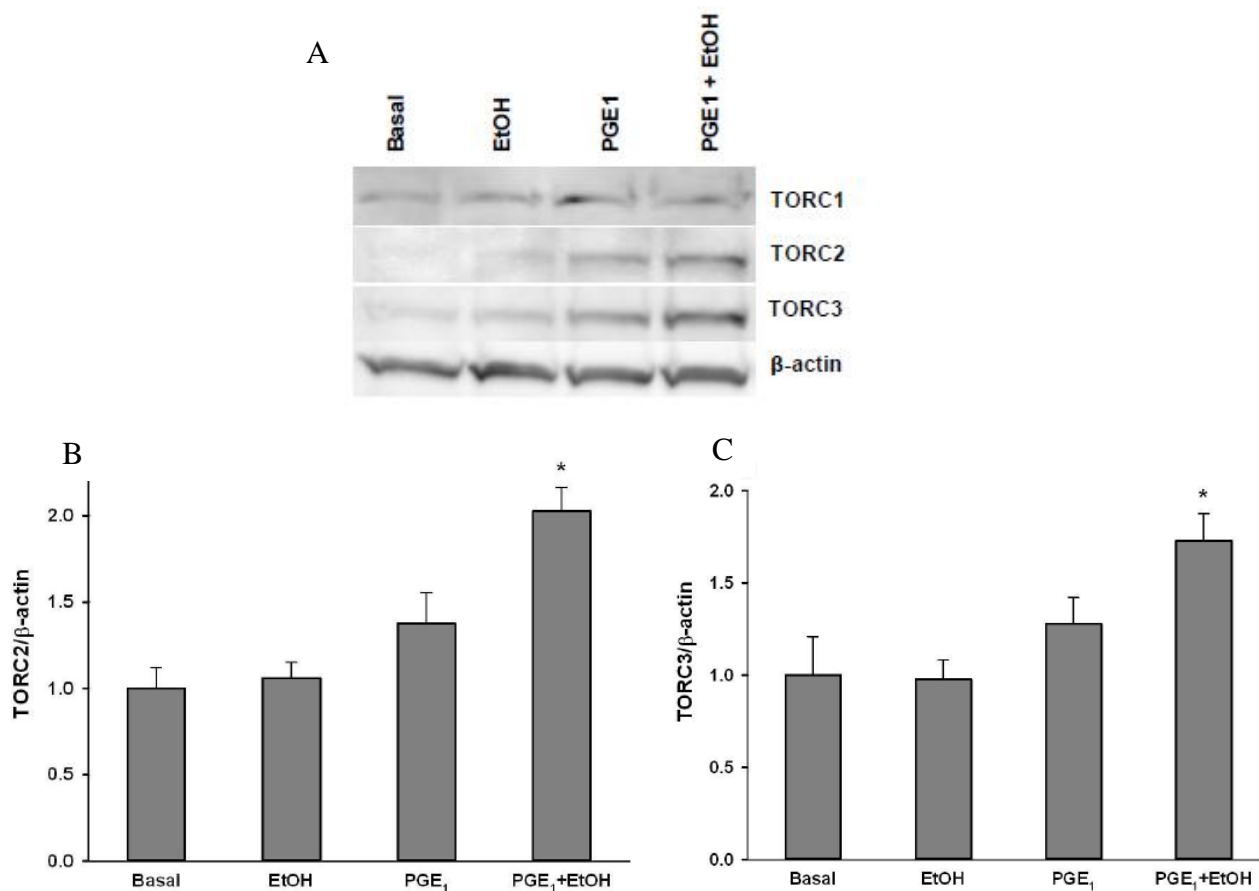


Figure 3.11. Immunodetection of TORC1, TORC2, and TORC3 in RAW 264.7 cells. (A) Cells were incubated with medium alone (basal), PGE<sub>1</sub> (10 μM), EtOH (150 mM), or PGE<sub>1</sub> +EtOH for 30 min. (B) Changes in TORC2 were normalized using β-actin (n = 4). (C) Changes in TORC3 were normalized using β-actin (n = 4). Analysis was similar to (B). \*Value significantly greater than basal (p < 0.05, 1-way ANOVA).

specific to TORC1 showed reduced nuclear translocation in BV-2 cells treated with PGE<sub>1</sub> and ethanol. The band intensities of TORC3 normalized against  $\beta$ -actin showed an enhancement of nuclear translocation in BV-2 cells treated with PGE<sub>1</sub> alone and PGE<sub>1</sub> in the presence of ethanol (Figure 3.12). The signal specific to TORC2 in BV-2 cells was too weak to be quantified (data not shown). The signal specific to TORC3 in nuclear extracts of cells treated with PGE<sub>1</sub> in the presence of ethanol showed a significant greater amount of TORC3 in RAW 264.7 cells compared to BV-2 cells (Figure 3.13).

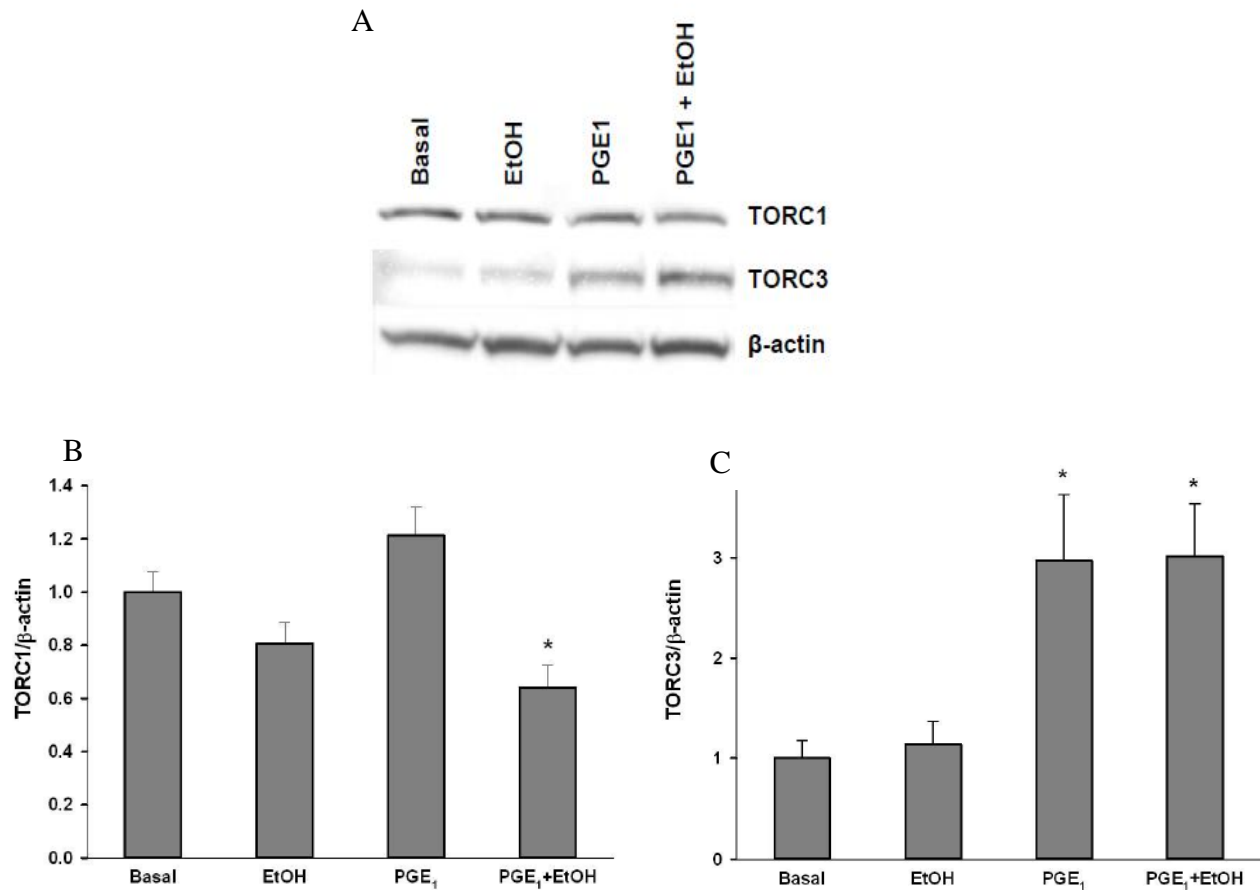


Figure 3.12. Immunodetection of TORC1 and TORC3 in BV-2 cells. (A) Cells were incubated with medium alone (basal), PGE<sub>1</sub> (10  $\mu$ M), EtOH (150 mM), or PGE<sub>1</sub> +EtOH for 30 min. (B) Changes in TORC1 were normalized using  $\beta$ -actin (n = 4). \*Value significantly greater than basal (p < 0.05, 1-way ANOVA). (C) Changes in TORC3 (n = 4). Analysis was similar to (B).

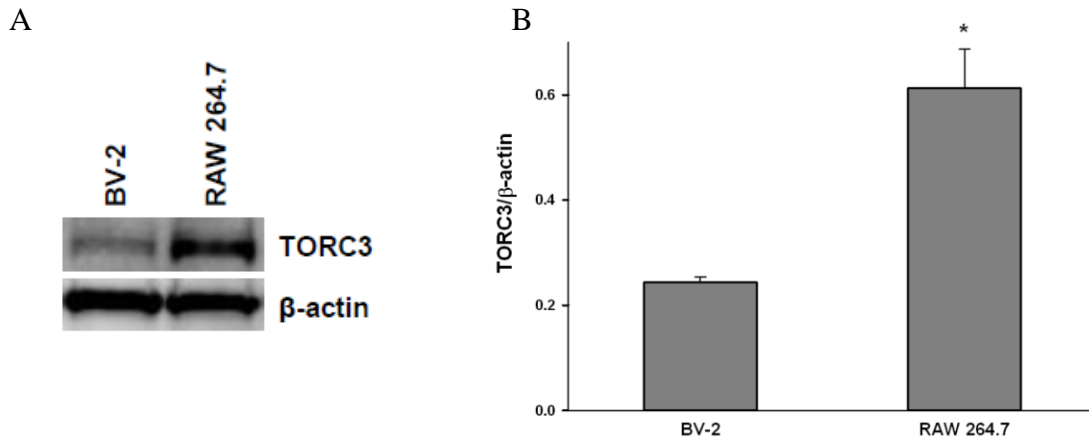


Figure 3.13. Immunodetection of TORC3 in RAW 264.7 and BV-2 cells. (A) Western blot analysis of nuclear extracts from BV-2 and RAW 264.7 cells. Cells were incubated with PGE<sub>1</sub> (10 μM) + EtOH (150 mM) for 30 min. (B) Quantification of TORC3 in RAW 264.7 and BV-2 cells. Amounts were normalized using β-actin (n = 3). \*Amount of TORC3 in RAW 264.7 cell nuclear extracts is significantly greater than that of BV-2 cells (p < 0.05, student t-test).

## Discussion

Previous research suggested that changes in inflammatory and immune responses can be induced by alcohol. Cyclic AMP has immunosuppressive effects on the body, but is also required for proper development of the immune system. The mechanisms responsible for cAMP and ethanol's effects on the immune system have not been fully identified. We hypothesized that the ethanol effect on cAMP regulated gene expression is due to a change in the cAMP amount present in the cells, which is most likely the result of the enhancement of AC7 activity by ethanol. RAW 264.7 and BV-2 cells expressing Epac1-cAMP were stimulated with PGE<sub>1</sub> in the presence or absence of ethanol. Cyclic AMP levels rapidly increased after the addition of PGE<sub>1</sub>. When ethanol was added together with PGE<sub>1</sub>, cAMP was further increased. Cyclic AMP levels in BV-2 cells stimulated with thrombin and sphingosine-1-phosphate, AC7 specific activators, followed a similar time course to that of cells stimulated with PGE<sub>1</sub> and ethanol.

Previous studies have demonstrated that AC7 is the dominant AC isoform expressed in RAW 264.7 cells (Jiang et al., 2007; Jiang et al., 2008). Cyclic AMP accumulation was evaluated in RAW 264.7 cells; however, these cells did not uptake enough  $^3\text{H}$ -labeled adenine to accurately measure cAMP accumulation (data not shown). A cAMP accumulation assay using a population of BV-2 cells showed enhancement of cAMP production during PGE<sub>1</sub> stimulation. This effect was further enhanced in the presence of ethanol, thrombin, and sphingosine-1-phosphate. Cyclic AMP accumulation was also significantly increased by ethanol at pharmacologically relevant concentrations of alcohol (25 and 50 mM) in BV-2 cells. These results are consistent with our previous observations in Hela, HEK 293, and NIH 3T3 cells which overexpressed AC7 by transfection (Gupta et al., 2013; Hill et al., 2016; Yoshimura and Tabakoff, 1995) and strongly suggest that BV-2 cells predominantly express AC7.

Cyclic AMP regulated gene expression was evaluated in RAW 264.7 and BV-2 cell lines. The Firefly luciferase activity in RAW 264.7 cells responded to cAMP stimulation as indicated above. However, Firefly luciferase and Renilla luciferase activity in BV-2 cells was too low to accurately quantify the effects of PGE<sub>1</sub> and ethanol. This result may be due to a low amount of transcription factor binding occurring upstream of the luciferase sequence, or low transfection efficiency in BV-2 cells. Firefly luciferase activity in RAW 264.7 cells showed that PGE<sub>1</sub> caused a concentration-dependent increase in enzymatic activity. Cyclic AMP regulated reporter gene expression in RAW 264.7 cells is affected by ethanol in a concentration-dependent and time-dependent manner. Firefly luciferase activity is significantly increased by ethanol at pharmacologically relevant concentrations in RAW 264.7 cells. The effect of ethanol on Firefly luciferase activity of RAW 264.7 cells reaches its maximum at 125 mM and decreases at 150

mM. This result is similar to the ethanol response of Firefly luciferase activity in NIH 3T3 cells expressing AC7 (Hill et al., 2016).

To further examine the effect of alcohol on cAMP regulated reporter gene expression, we uncoupled AC from the cAMP signaling pathway by using a cAMP analog, 8-bromo-cAMP. Cyclic AMP driven reporter gene expression was increased by 8-bromo-cAMP treatment in RAW 264.7 cells. However, treatment with the cAMP analog was not significantly affected by ethanol. This result indicates that the enhancement of cAMP regulated gene expression is due to increased AC activity caused by ethanol; ethanol does not significantly affect reactions occurring downstream of cAMP generation.

Studies have shown that incubation with ethanol increased the level of pCREB in NG108–15 cells (Constantinescu et al., 1999) and NIH 3T3 cells (Roman et al., 2005). However, in the current study the level of pCREB in nuclear extracts from RAW 264.7 and BV-2 cells were not significantly increased by stimulation with PGE<sub>1</sub>, ethanol, or PGE<sub>1</sub> and ethanol. Those results are similar to those observed in chapter 2 with NIH 3T3 cells overexpressing AC7. Changes in the phosphorylation state of CREB in response to cAMP pathway activation or ethanol treatment were not detectable. Similar to the NIH 3T3 cells in our laboratory, the basal level of Ser133 phosphorylation of CREB in the RAW 264.7 and BV-2 cells was very high. A variety of protein kinases, including PKA, can cause CREB phosphorylation (Johannessen et al., 2004). This indicates that besides PKA activation and Ser-133 phosphorylation, there may be additional protein kinases, co-activators, and transcription factors that are responsible for the diverse response of CREB phosphorylation.

Immunodetection of TORCs in RAW 264.7 cells showed that nuclear translocation of TORC2 and TORC3 are significantly enhanced by PGE<sub>1</sub> in the presence of ethanol. Nuclear



translocation of TORC1 in RAW 264.7 cells did not significantly change by stimulation with PGE<sub>1</sub> with or without ethanol. The changes of TORC2 and TORC3 appear to correlate with the changes in cAMP regulated reporter gene expression in RAW 264.7 cells. Immunodetection of TORCs in BV-2 cells showed that PGE<sub>1</sub> increased TORC3 translocation to the nucleus. TORC2 signals in BV-2 cells were too weak to quantify. Additionally, TORC3 in the nucleus appears significantly greater in RAW 264.7 cells than BV-2 cells.

In conclusion, our findings indicate that the ethanol effect on cAMP regulated reporter gene expression is due to the enhancement of endogenously expressed AC7 in RAW 264.7 cells. This enhancement leads to an increase in the amount of cAMP present in the cells. The alcohol effect observed is concentration- and time- dependent. These results show that ethanol's effect on AC activity plays a major role on the expression of a cAMP regulated reporter gene in RAW 264.7 and BV-2 immune cells. The increase in cAMP by addition of ethanol in RAW 264.7 is correlated with an increase in reporter gene activity as well as TORC2 and TORC3 nuclear translocation. The increase in cAMP by the addition of AC7 specific activators in BV-2 cells indicates that AC7 is the major AC isoform expressed in this cell line. Furthermore, an increase in cAMP in BV-2 cells is correlated with the increase in TORC3 translocation to the nucleus. Taken together, these results indicate that in addition to CREB, the TORC pathway may play a role in ethanol's effect on cAMP regulated gene expression and the AC7 isoform is critical for this response.

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## **CHAPTER 4. CONCLUDING REMARKS**

### **Overall Summary of Findings**

Research has established that alcohol has a clear effect on cAMP cell signaling. Alterations in cAMP signaling after exposure to alcohol have been observed in human cells as well as experimental animal models for alcohol use (Diamond et al., 1987; Donohue et al., 2005; Kirstein and Tabakoff, 2001; Nagy et al., 1988). Adenylyl cyclase, the enzyme that catalyzes the conversion of ATP to cAMP, is postulated to be the target of alcohol's effect on cAMP signaling (Yoshimura and Tabakoff, 1995). The main objective in our laboratory has been to characterize alcohol's effect on AC. To date, we have demonstrated that the activity of AC is enhanced by ethanol in an isoform-specific manner, AC7 is most enhanced by ethanol, and the ethanol responsive domains are located within the cytoplasmic regions of the AC protein (Dokphrom et al., 2011; Hasanuzzaman and Yoshimura, 2010; Kou and Yoshimura, 2007; Yoshimura et al., 2006; Yoshimura and Tabakoff, 1995; Yoshimura and Tabakoff, 1999). The current study expanded our research on the cAMP signaling pathway to include AC as well as downstream effectors, CREB and TORC.

In chapter 2, we transfected NIH 3T3 cells with specific AC isoforms to evaluate alcohol's effects on cAMP regulated gene expression. We optimized the experimental conditions of two AC isoforms, AC7 and AC3, in order to evaluate cAMP regulated gene expression in NIH 3T3 cells. We accomplished this by using an artificial GAL4-CREB fusion protein and a reporter gene containing GAL4 DNA binding sites situated upstream of a Firefly luciferase gene. We also manipulated various experimental conditions including incubation duration as well as dopamine, ethanol, and 8-bromo-cAMP stimulant concentrations. Our results determined that there is a clear ethanol effect on Firefly luciferase activity in AC7 expressing cells; this effect was not

observed in cells expressing AC3. Contrary to previous research, CREB phosphorylation in our NIH 3T3 cells was not affected by ethanol. This result was unexpected and led us to conclude that other factors play a more important role in alcohol's effect on cAMP regulated gene expression than changes in the phosphorylation state of CREB. One of these factors, TORC3, showed significant changes in nuclear translocation in cells treated with PGE<sub>1</sub> and ethanol.

In chapter 3, we evaluated the effect of alcohol on cAMP signaling and cAMP regulated gene expression in two immune cell lines. RAW 264.7 cells are a mouse macrophage cell line that primarily expresses AC7 (Jiang et al., 2007; Jiang et al., 2008). Messenger RNA expression of various AC isoforms has shown that AC7 is the dominant isoform in BV-2 mouse microglia cells (Atwood et al., 2011); our study is the first to confirm these results at a protein, functional level. BV-2 cells showed that intracellular cAMP is increased in the presence of varying AC7 stimulants including ethanol, thrombin, and sphingosine-1-phosphate. These results indicate that AC7 is endogenously expressed in BV-2 cells. The results also show that the effect of ethanol on cAMP in BV-2 cells is concentration-dependent. We have shown that alcohol has an enhancing effect on cAMP signaling as well as cAMP regulated gene expression in RAW 264.7 cells, which endogenously express AC7. The results in our RAW 264.7 cells and BV-2 cells are similar to the results obtained in chapter 2 in NIH 3T3 cells with overexpressed AC7. The effect of alcohol on cAMP regulated gene expression is time-dependent as well as PGE<sub>1</sub> and ethanol concentration-dependent in RAW 264.7 cells. CREB phosphorylation in these mammalian cell lines was not affected by alcohol, however, nuclear translocation of TORC2 and TORC3 was enhanced in RAW 264.7 cells and nuclear translocation of TORC3 was enhanced in BV-2 cells. There was no significant difference in the nuclear translocation of TORC3 in BV-2 cells by the addition of alcohol and PGE<sub>1</sub> compared to PGE<sub>1</sub> alone. Interestingly, when comparing the two

immune cell lines, BV-2 cells had significantly less TORC3 nuclear translocation in cells stimulated with PGE<sub>1</sub> in the presence of ethanol than RAW 264.7 cells that were stimulated with the same conditions, indicating that there may be a substantial difference between the basal transcription machinery of these two cell lines.

### **Significance of Research**

Adenylyl cyclase catalyzes the conversion of ATP to cAMP. The activation of the cAMP signaling cascade initiates many downstream cellular events including cell growth, cell differentiation, cytokine production, glycolysis, and gene expression. This cascade has also been implicated in learning as well as in short term and long term memory formation. The effect of alcohol on AC may have downstream effects on these cAMP regulated functions. Therefore, understanding how alcohol modulates AC may assist in explaining the physiological consequences, including changes in immune function, of acute and chronic alcohol exposure.

While research on the clinical and experimental effects of alcohol continues to expand, there is a clear gap in the knowledge in regards to cAMP regulated gene expression and immune function. Much of the research regarding alcohol, cAMP, and the immune system appears to be conflicting. For example, cAMP has an immunosuppressive effect, but is also required for proper development of the immune system and immune responses (Boer et al., 2003; Castro et al., 2005; Mosenden and Taskén, 2011; Sanders, 2012). Furthermore, it has been shown that immune responses are reduced in patients who consume high-doses of alcohol, but low-doses of ethanol may enhance the immune response (Cook, 1998; Romeo et al., 2007).

We are the first to investigate the effects of alcohol and AC isoform specificity on cAMP regulated gene expression. By utilizing the AC isoform most responsive to alcohol, which is also highly expressed in immune cells, we may be able to elucidate the mechanism through which

alcohol affects downstream cellular events in the cAMP pathway including gene expression, CREB phosphorylation, and nuclear translocation of TORC. These studies have great implications for the field of alcohol research and may assist in the development of clinical tools for the diagnosis and treatment of alcohol-related diseases and disorders.

### **Future Directions**

The results presented in this dissertation provide clear evidence that alcohol enhances cAMP regulated gene expression through enhancement of AC activity, with AC7 being most alcohol responsive isoform. The next milestone in this research will be to generate an AC7 knockout cell line from immune cell lines, RAW 264.7 cells and BV-2 cells, using CRISPR-Cas9 mediated genome editing. By generating an AC7 knockout cell line, we can more precisely evaluate AC7's role on alcohol's effects on cAMP production as well as gene expression and immune function. Presently, we have designed a 20-nucleotide long AC7 specific gRNA from the mouse and human AC7 genomic sequence. The primary limiting factor for the accomplishment of this step appears to be the low transfection efficiency of our surrogate reporter gene. Single cell isolation using fluorescence-activated cell sorting has also shown to be a challenge. Once the AC7 knockout cell line is generated and alcohol's effects on gene expression as well as immune function have been evaluated, we plan to expand this research by utilizing a conditional AC7 knockout mouse provided by the University of Texas Southwestern Medical Center.

In conclusion, these studies have provided the means for examining the effect of alcohol on cAMP regulated gene expression. We have begun to elucidate the potential mechanisms for the interaction of AC and alcohol; the results presented herein provide a solid foundation for



future studies that may have the capability to resolve the exact role of alcohol on adenylyl cyclase and its potential effects on the immune system.

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