



The effect of alcohol on recombinant proteins derived from mammalian adenylyl cyclase



Emily Qualls-Creekmore¹, Ratna Gupta², Masami Yoshimura*

Department of Comparative Biomedical Sciences, School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA 70803, United States

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ABSTRACT

The cyclic AMP (cAMP) signaling pathway is implicated in the development of alcohol use disorder. Previous studies have demonstrated that ethanol enhances the activity of adenylyl cyclase (AC) in an isoform specific manner; AC7 is most enhanced by ethanol, and regions responsible for enhancement by ethanol are located in the cytoplasmic domains of the AC7 protein. We hypothesize that ethanol modulates AC activity by directly interacting with the protein and that ethanol effects on AC can be studied using recombinant AC *in vitro*. AC recombinant proteins containing only the C_{1a} or C₂ domains of AC7 and AC9 individually were expressed in bacteria, and purified. The purified recombinant AC proteins retained enzymatic activity and isoform specific alcohol responsiveness. The combination of the C_{1a} or C₂ domains of AC7 maintained the same alcohol cutoff point as full-length AC7. We also find that the recombinant AC7 responds to alcohol differently in the presence of different combinations of activators including MnCl₂, forskolin, and Gsa. Through a series of concentration-response experiments and curve fitting, the values for maximum activities, Hill coefficients, and EC₅₀ were determined in the absence and presence of butanol as a surrogate of ethanol. The results suggest that alcohol modulates AC activity by directly interacting with the AC protein and that the alcohol interaction with the AC protein occurs at multiple sites with positive cooperativity. This study indicates that the recombinant AC proteins expressed in bacteria can provide a useful model system to investigate the mechanism of alcohol action on their activity.

1. Introduction

There are nine membrane-bound isoforms and one soluble isoform of mammalian AC. Various isoforms of AC are expressed differentially among tissue type and organ systems [1]. AC isoforms are classified based on the structural homology and the variation in regulatory mechanisms; primary regulators of AC activity include G proteins, calcium, calmodulin, protein kinase C, and small molecules such as forskolin [1,2]. The structure for membrane-bound AC consists of a cytoplasmic N-terminal domain (N), followed by a membrane-spanning domain (M₁), a cytoplasmic domain (C_{1a} and C_{1b}), a second membrane-spanning domain (M₂), and a second cytoplasmic domain (C_{2a} and C_{2b}) [3]. The C_{1a} and C_{2a} domain are homologous and constitute the catalytic core [3]. The cytoplasmic domains are also where key regulators of AC interact with the protein, including G protein subunits as well as small molecule activators of AC such as forskolin [1].

Both human and model animal studies have implicated the cAMP signaling system in the development of and predisposition to alcohol use disorder. Many of these studies have implicated AC as a crucial component of the ethanol effect on cAMP signaling. For example, complete abstainers from alcohol but with a family history positive for alcoholism had lower basal and stimulated AC activity levels than complete abstainers with no family history of alcoholism [4], suggesting AC activity as a possible trait marker for genetic predisposition for the development of alcoholism. Genetically modified mice have been employed to study the role of the cAMP signaling system in alcohol related behaviors. Double knock-out (DKO) mice lacking both AC1 and AC8 had increased sensitivity to alcohol-induced sedation, further, DKO mice and AC8 KO mice exhibit decreased voluntary ethanol consumption compared to wild type mice [5]. AC5 KO mice show higher preference for ethanol as well as increased total consumption of ethanol compared to wild type mice [6]. Another transgenic mouse study found

Abbreviations: AC, Adenylyl cyclase; cAMP, Cyclic AMP; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DKO, Double knock out; KO, Knock out; PMSF, phenylmethylsulfonyl fluoride; GppNHp, Guanosine 5'-[β,γ-imido]triphosphate

* Corresponding author.

E-mail address: fukugawa@lsu.edu (M. Yoshimura).

¹ Present address: Pennington Biomedical Research Center, Louisiana State University System, Baton Rouge, LA 70808, United States.

² Present address: School of Arts and Sciences, Our Lady of the Lake College, Baton Rouge, LA 70808, United States.

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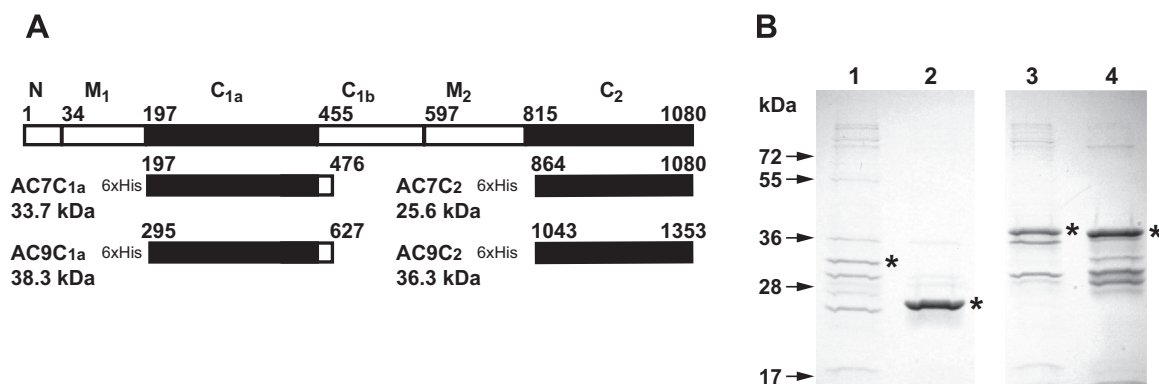


Fig. 1. A) Schematic representation of recombinant AC constructs. The top figure represents the structure of the native form of AC7. The numbers indicate the positions of the amino acid residues in the respective native isoform. The positions of recombinant proteins are shown with their predicted molecular weights. 6xHis indicates a hexahistidine tag. B) SDS-PAGE analysis of purified recombinant AC proteins. Lane 1; AC7 C_{1a}, Lane 2; AC7 C₂, Lane 3; AC9 C_{1a}, Lane 4; AC9 C₂. Asterisks indicate the bands detected by Western blotting with His-tag monoclonal antibody.

that overexpression of AC7 in mice increased basal and ethanol-induced DARPP-32, a downstream element of the cAMP pathway implicated in motivation for ethanol intake [7].

We have demonstrated that ethanol enhances the activity of AC in an isoform specific manner and that the isoform AC7 is most enhanced by ethanol as measured by cAMP accumulation assay in intact mammalian cells, including HEK 293, HeLa, and NIH 3T3 cells [8–10]. Ethanol responsive domains of AC7 were shown to be localized in the cytoplasmic regions of the protein [11]. We found that different AC isoforms have different responses to a series of *n*-alkanols; AC6, AC7, and AC9 were found to have different alcohol cut-off points [12]. Subsequent study found that 2,3-butanediol had effects on AC activity not only is an AC isoform specific manner but also in a stereoisomer specific manner [13]. These studies suggest that alcohol exhibits its effect on AC activity by directly interacting with AC protein. However, the experimental system employed in these studies, transfected mammalian cells, is not suitable to demonstrate this notion. Therefore, we decided to pursue AC recombinant proteins expressed in bacteria as an experimental system for investigating the specific interactions between alcohol and AC. We generated a recombinant AC7 protein consisting of the C_{1a} and C₂ domains linked together. This protein, named AC7sol, which included the ethanol responsive domains identified previously, was expressed in a strain of *E. coli* lacking AC activity and lysate preparation was assayed for enzymatic activity devoid of any other mammalian proteins. We found that AC activity was enhanced by alcohols in a manner consistent with our previous mammalian studies and demonstrated that in crude bacterial lysate, the C_{1a} and C₂ domains are sufficient to produce the alcohol effect [14]. This study, however, did not demonstrate the isoform specificity of alcohol effect.

In the present study, we constructed and purified AC recombinant proteins containing one of the cytoplasmic domains, the C_{1a} and C₂ domains, derived from AC7 and AC9, to further examine the hypothesis that alcohol modulates AC activity by directly interacting with the AC protein. We selected AC9 as the second isoform in this study because this isoform has smaller ethanol response and different alcohol cutoff point compared to AC7 [12]. In addition, successful expression of recombinant proteins derived from AC9 was reported [15]. The isoform specificity of alcohol effect and the role of each cytoplasmic domain in the effect of alcohol were examined. In addition, we attempted to elucidate the regulatory mechanism by which alcohol modulates AC activity in the presence of MnCl₂, forskolin, and Gα in a series of concentration-response experiments.

2. Materials and methods

2.1. Reagents

Restriction enzymes, Phusion Hot Start DNA polymerase, and Quick Ligation Kit were from New England Biolabs (Ipswich, MA). Bacto Tryptone and Bacto Yeast extract were from BD (Franklin Lakes, NJ). HP ampicillin was from Geno Technology (Maryland Height, MO). HisPur Cobalt chromatography cartridges were from Pierce Biotechnology (Rockford, IL). HiTrap SP HP and HiTrap Q HP ion exchange chromatography cartridges were from GE Healthcare (Piscataway, NJ). His-Tag monoclonal antibody was from Novagen by EMD Millipore (Darmstadt, Germany). BCA protein assay kit was from Pierce Biotechnology (Rockford, IL). Calbiochem Protease Inhibitor Cocktail II was from EMD Millipore (Darmstadt, Germany). [³H] cAMP was from Moravsek Biochemicals (Brea, CA). [α-³²P] ATP was from Perkin Elmer (Boston, MA). All other chemicals and reagents used were from Sigma-Aldrich (St. Louis, MO).

2.2. Construction of recombinant AC

Recombinant proteins were designed to include the ethanol responsive domains previously identified in the native AC7 protein [11]. AC9 recombinants were designed to include the regions homologous to those employed for the AC7 recombinant proteins (Fig. 1A). The N-terminal tail, transmembrane-spanning domains, and C_{1b} regions were excluded from the recombinant proteins. All recombinant proteins included a hexahistidine tag for purification purposes. DNA fragments were amplified by polymerase chain reaction (PCR) from their respective template DNA (Human AC7, Gen Bank ID D25538; Mouse AC9, Gen Bank ID MN009624), using Phusion Hot Start DNA polymerase and two oligonucleotide primers. The resulting PCR products were digested with *Bam*HI and *Hind*III and ligated into pQE82L (Qiagen, Valencia, CA) also digested by *Bam*HI and *Hind*III. Resulting plasmids were designated as pQE82L AC7 C_{1a}, pQE82L AC7 C₂, pQE82L AC9 C_{1a}, and pQE82L AC9 C₂. The coding sequence of each recombinant protein was confirmed by DNA sequencing analysis.

2.3. Expression of recombinant AC and Gα

Plasmids carrying AC coding sequences prepared in the previous section and a plasmid carrying Gα coding sequence [16] were transformed in an *E. coli* expression system using cell lines BL21 (DE3) carrying plasmid pUSB520, and grown overnight on LB plates containing appropriate antibiotics. The next day, colonies were harvested and inoculated in T7 medium with antibiotics required for the plasmid and cell line and grown at 30 °C at 250 rpm until the A600

reached 2. This seed culture was then inoculated in 4 L T7 medium plus antibiotics and continued to grow at 30 °C at 250 rpm. Protein expression was induced at A600 value of 0.6 with 100 μM of isopropyl β-D-1-thiogalactopyranoside. Following induction, the culture was grown for 19 h at 25 °C at 100 rpm. The bacteria were then harvested by centrifugation and were stored at –80 °C.

2.4. Purification of recombinant AC and Gsa

The purification procedure modified from published method [16,17] was carried out at 4 °C. Cells were suspended in cold lysis buffer (20 mM Tris-HCl pH 7.4, 5 mM βmercaptoethanol, 100 μM phenylmethylsulfonyl fluoride (PMSF), 22 μg/ml *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone, 22 μg/ml *N*-α-tosyl-L-lysine chloromethyl ketone hydrochloride, trypsin inhibitor, and Sigma Protease Inhibitor Cocktail (1 tablet/100 ml) and lysed by sonication in the presence of lysozyme (1 mg/ml) on ice. Soluble proteins were separated by ultracentrifugation using Beckman Type 50.2 Ti rotor at 45,000 rpm for 30 min at 4 °C and loaded onto HisPur Cobalt chromatography cartridge, washed with the lysis buffer containing 500 mM NaCl, followed by the same buffer containing 100 mM NaCl and 10 mM Imidazole until A280 decreased to a plateau. Proteins were eluted into 20 fractions using an imidazole gradient 10–500 mM in the lysis buffer. Eluted fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fractions containing the target protein were pooled for subsequent purification by ion exchange chromatography. The pooled fractions were diluted by 5 volumes in loading buffer (20 mM Tris-HCl pH 7.4, 1 mM dithiothreitol, 500 μM ethylenediaminetetraacetic acid (EDTA), Calbiochem Protease Inhibitor Cocktail, and 100 μM PMSF) and loaded to the HiTrap Q-HP or HiTrap SP-HP 5 ml column depending on the charge of the target protein. The column was washed with the loading buffer and proteins were eluted into 50 fractions using a NaCl gradient 0–1 M. Eluted fractions were again analyzed by SDS-PAGE and fractions containing the target protein were pooled. Pooled fractions were concentrated using Amicon Ultra 10 K filters under centrifugation at 4 °C and stored at –80 °C.

Purified proteins were separated by SDS-PAGE and stained with Bio-safe coomassie stain (BioRad). The proteins were transferred to nitrocellulose membranes for western blot analysis. Western-blotting used Anti-His monoclonal antibody to identify proteins containing a 6 histidine tag at the N-terminus of the protein construct. Briefly, membranes were blocked with blocking buffer containing 1% milk in tris-buffered saline (20 mM Tris HCl pH 7.5, 150 mM NaCl) with 0.2% tween 20 (TBST) for 1 h at room temperature. Following washing, membranes were incubated with Anti-His antibody 1:1000 dilution in the blocking buffer overnight at 4 °C. Membranes were washed and incubated with anti-mouse secondary antibody conjugated horseradish peroxidase, 1:5000 dilution. Blots were developed using ECL detection reagents (GE Healthcare, Piscataway, NJ). Protein quantity was estimated using the BCA protein assay kit or based on their molecular extinction coefficient at 280 nm.

2.5. Enzymatic activity assay

The total reaction mixture of 200 μl contained 5–20 μg of total purified AC proteins (one C_{1a} protein and one C₂ protein combined), 250 μM ATP, 2.5 mM MgSO₄, 25 mM Tris-HCl pH 7.4, 100 μM 3-isobutyl-1-methylxanthine, 10 mM phosphocreatine, 5 U creatine phosphokinase, and [α-³²P] ATP (50–80 cpm/pmol). AC activity was stimulated in the presence of activators as indicated. When Gsa was used, it was activated with Guanosine 5'-[β,γ-imido]triphosphate (GppNHp) and 3.5 mM MgSO₄ as described previously [14]. Reaction mixtures were prepared on ice and the assay was initiated by temperature-shift to 30 °C for 15 min. The reaction was terminated by the addition of a 200 μl solution containing 2% SDS, 1 mM ATP, and 1 mM cAMP. ³H cAMP was added to estimate recovery of cAMP.

Sequential column chromatography was used to separate [α-³²P] ATP and [α-³²P] cAMP by dowex 50 and neutral alumina columns. A liquid scintillation counter was used to detect radioactivity in the final sample [18].

2.6. Data analysis

All assay data were acquired in triplicate and presented as mean ± SD in the graphs. Each experiment was performed at least twice to ensure reliability of measures. Concentration response curve fitting was carried out by non-linear regression analysis using either 3 or 4 parameter logistic equation:

$$y = \frac{a}{1 + \left(\frac{x}{x_0}\right)^b} \text{ or } y = y_0 + \frac{a - y_0}{1 + \left(\frac{x}{x_0}\right)^b}$$

a: maximum value, b: Hill coefficient, x₀: EC₅₀, y₀: minimum value. Statistical analysis was carried out either by *t*-test or one-way ANOVA with pair wise comparisons by the Holm–Sidak method. In both cases, significance was determined by p-value < 0.05. Sigma Plot and Sigma Stat (Systat Software, Point Richmond, CA) were used to plot and analyze data.

3. Results

3.1. Protein production

The expression and purification of recombinant AC proteins was successful for each construct; however, there was variation in the quality of the final product. Expression of AC7 C_{1a} and AC9 C_{1a} was lower in comparison to their AC7 C₂ and AC9 C₂ counterparts (Fig. 1B). The elution profile for AC7 C_{1a} showed a wider peak and more contamination by other proteins. AC9 C_{1a} followed a similar pattern as AC7 C_{1a} with modest expression, though slightly higher than AC7 C_{1a}. AC7 C₂ presented high levels of expression and only minor contamination following purification. AC9 C₂ also had high levels of expression; however, the purification of this protein resulted in some non-specific protein bands similar to results by others producing a recombinant version of AC9 C₂ [15].

3.2. Enzymatic activity of recombinant AC

The initial enzymatic activity assay was carried out using AC7 C_{1a} alone, AC7 C₂ alone, and AC7 C_{1a} and AC7 C₂ combined. For each preparation, AC activity was stimulated with 2.5 mM MnCl₂. We found that when assayed alone, C_{1a} or C₂ had very small activity. However, the combined preparation of C_{1a} and C₂ showed high levels of AC activity (Fig. 2). These results demonstrated that even though the catalytic components, C_{1a} and C₂, are expressed and purified separately, they can be combined *in vitro* and enzymatic activity is restored. We observed a similar activity pattern with the AC9 recombinant proteins (data not shown).

3.3. Isoform specific ethanol effect

We first examined the effect of ethanol on isoforms AC7 and AC9 using homologous combinations of their respective C_{1a} and C₂ recombinant proteins. AC7 C_{1a} + AC7 C₂ and AC9 C_{1a} + AC9 C₂ combinations were assayed MnCl₂ plus or minus 200 mM ethanol. (Fig. 3A and B). Both protein combinations were active when stimulated by MnCl₂ alone. Compared to the basal activity, the activity stimulated with MnCl₂ was about 300 fold higher for AC7 and about 3000 fold higher for AC9. There was no significant difference between AC activity in the presence of MnCl₂ alone or MnCl₂ + forskolin for either AC7 or AC9. In the presence of ethanol, we observed a significant enhancement of AC7 activity (Fig. 3A), while the AC9 was significantly inhibited by ethanol

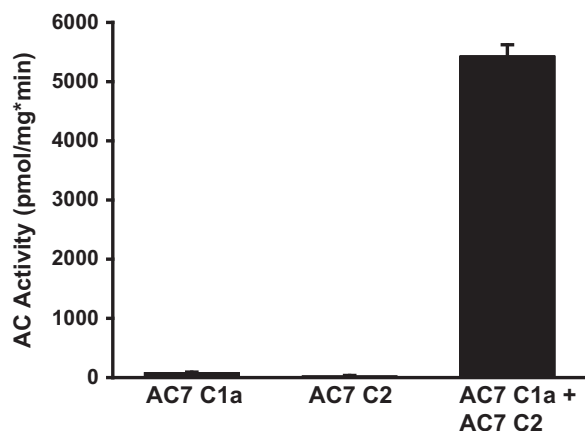


Fig. 2. Enzyme activity of AC7 recombinant proteins. AC activity of recombinant AC7 C_{1a} and AC7 C₂ alone, and AC7 C_{1a}+AC7 C₂ combined preparation are shown. AC activity was measured in the presence of 2.5 mM MnCl₂.

(Fig. 3B).

3.4. Ethanol effect on chimeric AC

In order to examine the role of each cytoplasmic domain, we determined the AC activity of 4 different combinations of cytoplasmic domains from AC7 and AC9 (Fig. 4A). Two combinations were homologous (AC7 C_{1a}+AC7 C₂ and AC9 C_{1a}+AC9 C₂) and two were chimeric (AC7 C_{1a}+AC9 C₂ and AC9 C_{1a}+AC7 C₂). All of the combinations were active, however, the specific activity for each of these combinations were quite diverse.

Ethanol enhanced the activity of AC7 C_{1a}+AC7 C₂ while inhibited the activity of AC9 C_{1a}+ AC9 C₂ (Fig. 4B). Ethanol did not show a significant effect on two chimeric AC's (AC7 C_{1a}+AC9 C₂ and AC9 C_{1a}+AC7 C₂).

3.5. Activation of AC and effect of butanol

The effect of AC activators (forskolin, MnCl₂, and Gsα) on AC7 C_{1a}+AC7 C₂ was examined in the presence of one activator, two, or all three (Fig. 5A). A series of structural studies of AC5 C1a and AC2 C2 domain indicated that these three activators have discrete binding sites on AC proteins and that their activation mechanisms are different from each other [19,20]. Therefore, we expected that the alcohol effect could be different depending on which activator or which combination of the activators is used. The AC activity under the basal condition and the activity in the presence of forskolin alone were very small and not significantly different from each other. AC activity in the presence of

Gsα alone or MnCl₂ alone was significantly greater than basal or forskolin-stimulated AC activity. Synergistic increase of AC activity was evident in the presence of Gsα + forskolin or MnCl₂ + Gsα compared to the activity stimulated by individual activator. However, MnCl₂ + forskolin did not increase AC activity compared to MnCl₂ alone. The results were consistent with the observation of AC7sol activity in bacterial lysate [14]. The combination of all three AC activators, MnCl₂ + forskolin + Gsα, also had a synergistic effect on AC activity.

We next examined the effect of 100 mM butanol on AC activity. Butanol was used as surrogate for ethanol. Our previous studies indicated that butanol has greater effect than ethanol on native form of AC7 in live cells and also on AC7sol expressed in bacteria [12,14]. Several studies showed that a series of *n*-alkanols had a similar effect on the function of proteins, including AC, NMDA receptors, GABA_A receptors, and glycine receptors [12,21,22], suggesting that *n*-alcohols have their effects on a given protein through interaction at common sites on the protein. The binding sites for alcohol were determined for some proteins, including LUSH and Munc13-1 from *Drosophila*, PKCδ and PKCε [23–26]. In each case, different *n*-alkanols bind to the similar if not the same sites. Therefore, it is reasonable to use butanol as surrogate for ethanol. The conditions for AC activation not only affected the magnitude of the butanol effect, but also determined if the effect of butanol was positive or negative on AC activity (Fig. 5B). The conditions resulting in enhancement of AC activity by butanol were MnCl₂ alone and MnCl₂+forskolin. All other combinations of AC activators resulted in AC inhibition when in the presence of 100 mM butanol; the extent of inhibition varied depending on the combination of AC activators.

3.6. Alcohol cutoff point of AC7 C_{1a}+AC7 C₂

Because many of the three activator combinations resulted in the inhibition of AC activity when exposed to butanol, we determined the alcohol cutoff point for AC7 C_{1a}+AC7 C₂ in the presence of MnCl₂, forskolin, and Gsα to see if the inhibition followed the same alcohol cutoff seen in native AC7 and recombinant AC7sol [12,14]. Alcohol cutoff point is defined as an alcohol in an ascending series of *n*-alkanols beyond which there is no further increase in the potency. AC activity was assayed in the presence of various concentrations of ethanol, butanol, pentanol, or hexanol (Fig. 6). Under these conditions, the potency of alcohol to inhibit AC activity increased until pentanol. Inhibition of AC activity by hexanol was no greater than inhibition by pentanol, suggesting that the alcohol cutoff was pentanol. This is consistent with our previous observations in native AC7 and AC7sol and provides rationale for use of these AC activation conditions in concentration response studies of recombinant AC7 C_{1a}+AC7 C₂.

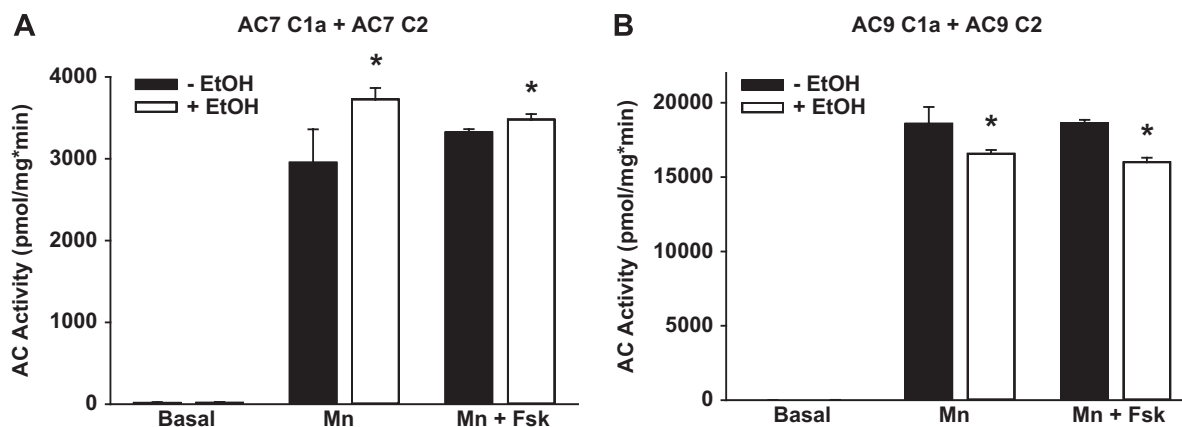


Fig. 3. Effects of ethanol on recombinant AC proteins. A) The activity of AC7 C_{1a}+AC7 C₂ was examined in the absence or presence of 200 mM ethanol. Assay conditions are: Basal; no additional stimulants, Mn; 2.5 mM MnCl₂, Mn + Fsk; 2.5 mM MnCl₂+10 μM forskolin. B) Same as A) for AC9 C_{1a}+AC9 C₂. *: Ethanol's effect is significant (*t*-test, *p* < 0.05).

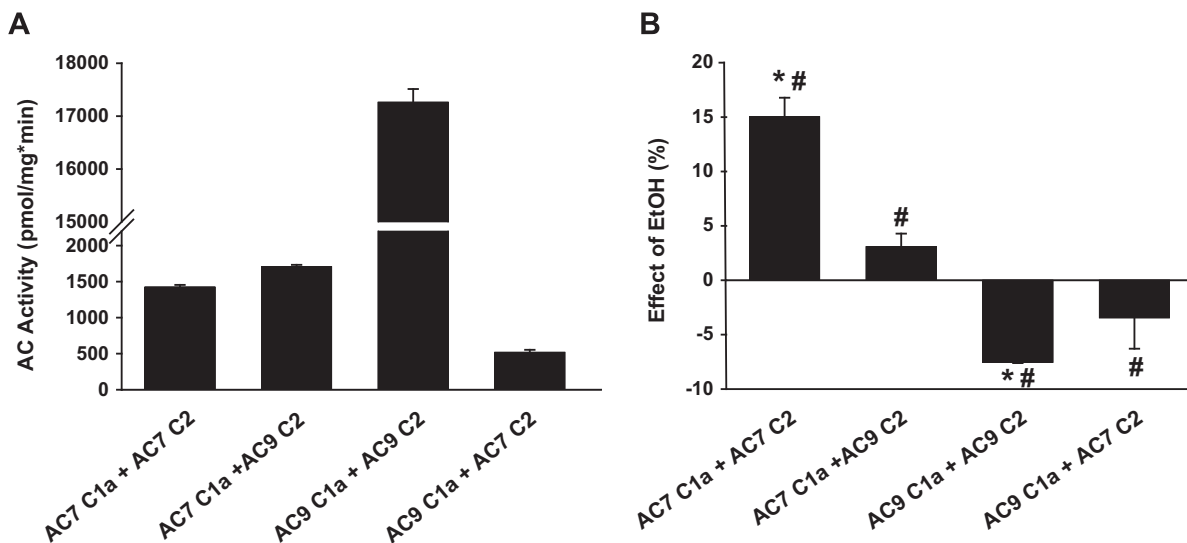


Fig. 4. Effects of ethanol in different combinations of C_{1a} and C₂ recombinant proteins. A) AC activities examined in the presence of 2.5 mM MnCl₂ are shown. B) Effects of 200 mM ethanol on MnCl₂ stimulated AC activity are shown. *: Ethanol's effect is significant (t-test). #: values are significantly different from each other (one-way ANOVA and pair wise comparison).

3.7. Interaction of butanol and AC activators

We carried out a series of concentration response experiments and curve fitting for the activity of AC7 C_{1a}+AC7 C₂. The concentration of MnCl₂ remained constant at 2.5 mM; concentrations of forskolin, GppNHp, activator of Gsα, and butanol varied by experiment.

Our first set of experiments explored the relationship between butanol and forskolin. In order to address the solubility constraints of forskolin, we used a water soluble forskolin analogue, NKH 477. Concentration-response experiments were performed using 2.5 mM MnCl₂ and 4 μg Gsα in the absence or presence of 100 mM BuOH (Fig. 7A). NKH 477 increased the activity of AC7 C_{1a}+AC7 C₂ in a concentration-dependent manner. Butanol decreased the AC activity at every concentration of NKH 477. Butanol significantly changed maximum value and EC50, while Hill coefficient remained constant (Table 1). Next we performed a concentration-response experiment using butanol ranging from 0 to 400 mM in the absence or presence of forskolin (Fig. 7B). Butanol decreased the AC activity in a concentration-dependent manner. The addition of forskolin significantly changed maximum value and EC50 (Table 2). Similar to NKH 477 concentration

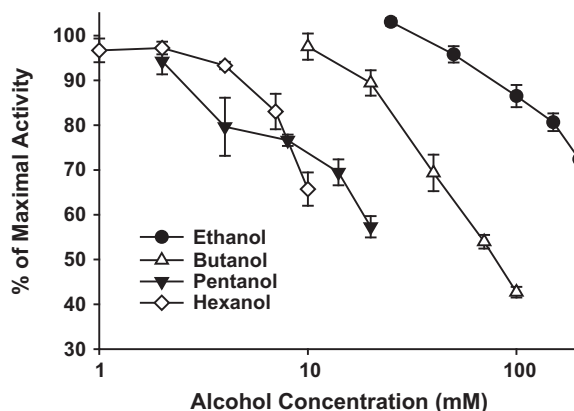


Fig. 6. Alcohol cutoff for AC7 C_{1a}+AC7 C₂. The activity of AC7 C_{1a}+AC7 C₂ was examined with 2.5 mM MnCl₂, 10 μM forskolin, and 4 μg activated Gsα in the presence of various concentrations of different n-alkanols as indicated.

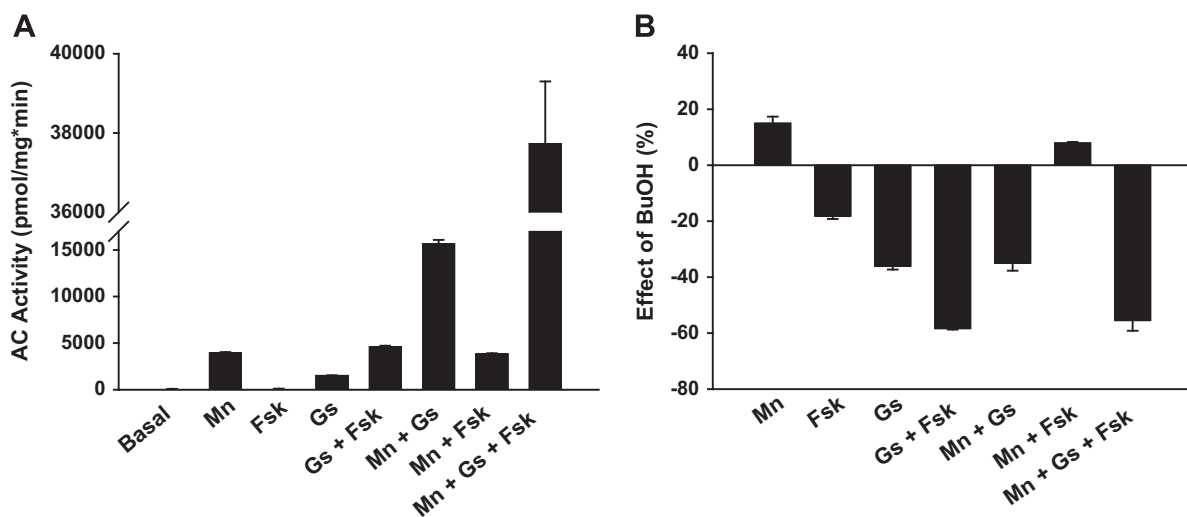


Fig. 5. Effects of butanol on the activity of AC7 C_{1a}+AC7 C₂. A) The activities of AC7 C_{1a}+AC7 C₂ are shown. Conditions are: Basal; no stimulants, Mn; 2.5 mM MnCl₂, Fsk; 10 μM forskolin, Gs; 4 μg activated Gsα. B) Effect of 100 mM butanol on AC7 C_{1a}+AC7 C₂ activity under various conditions of AC activation.

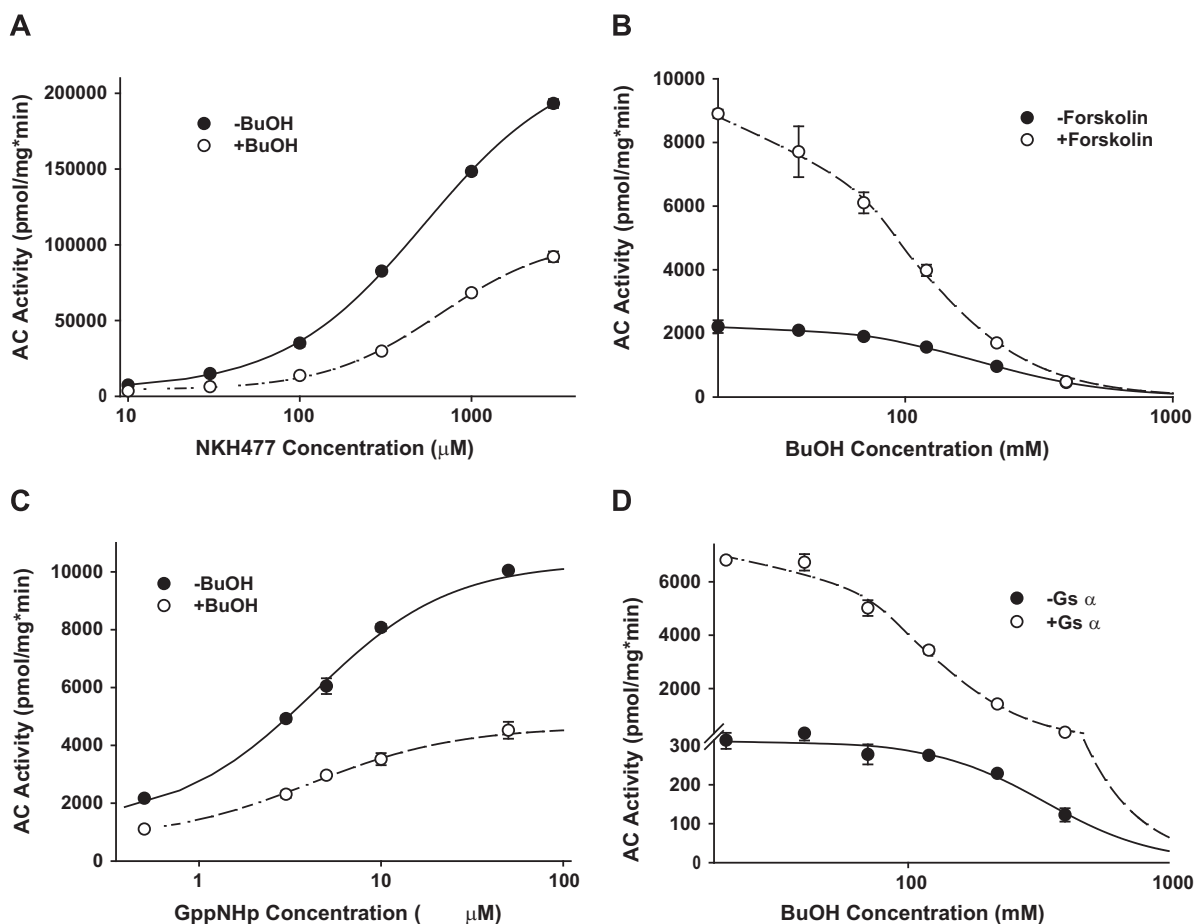


Fig. 7. Concentration dependent curves for the activity of AC7 C_{1a} + AC7 C₂. The curves were obtained by varying one of activators or butanol using regression analysis as described in Materials and Methods. Unless otherwise indicated, the reactions contained 2.5 mM MnCl₂, 25 μM forskolin, 4 μg activated Gsα, and 5 μg AC7 (C_{1a} + C₂). **A)** Forskolin analogue, NKH477, concentration response curve in the presence or absence of 100 mM butanol. **B)** Butanol concentration response curve in the presence or absence of 25 μM forskolin. **C)** GppNHp concentration response curve in the presence or absence of 100 mM butanol. **D)** Butanol concentration response curve in the presence or absence of activated Gsα.

response curves, Hill coefficient remained constant.

The interaction between Gsα and butanol was examined next. We performed a GppNHp concentration-response experiment (Fig. 7C). In this experiment we varied the concentration of GppNHp in the activation of Gsα while keeping the amount of Gsα constant. In the AC assay reaction, Gsα protein remained constant at 4 μg per reaction, along with 2.5 mM MnCl₂ and 25 μM forskolin. GppNHp increased AC activity of AC7 C_{1a} + AC7 C₂ concentration dependently, which indicated that the AC activity was increased in response to the concentration of activated Gsα. The presence of butanol did decrease AC activity. Butanol significantly decreased the maximum value, however, Hill coefficient and EC₅₀ remained constant (Table 1). When we performed a concentration-response experiment using butanol ranging from 0 to 400 mM in the presence or absence of Gsα (Fig. 7D), maximum value and EC₅₀ changed significantly in the presence of Gsα, while Hill coefficient remained constant (Table 2).

Table 1
Regression associated parameters and effects of butanol.

	Fig. 7A		Fig. 7C			
	maximum value	Hill coefficient	EC ₅₀	maximum value	Hill coefficient	EC ₅₀
-BuOH	$2.19 \times 10^5 \pm 5.74 \times 10^3$	-1.06 ± 0.05	534 ± 27	8980 ± 213	-1.16 ± 0.08	4.27 ± 0.21
+BuOH	$1.01 \times 10^5 \pm 4.42 \times 10^{3a}$	-1.28 ± 0.10	659 ± 53^a	3940 ± 201^a	-1.06 ± 0.14	4.05 ± 0.46

The parameters were obtained by regression analysis as described in Materials and Methods. Units used are pmol/mg protein* min for maximum values and μM for EC₅₀.

^a Significantly different from corresponding values shown directly above (*t*-test).

4. Discussion

Soluble recombinant AC proteins consisting of the catalytic domain of the enzyme (the C_{1a} or C₂ domain) have been valuable tools to elucidate the mechanisms by which the activity of AC is regulated by its regulators including Gsα, forskolin, Mg²⁺, and Ca²⁺ [1,27–29]. These recombinant proteins were also used for determination of 3D structure of AC catalytic domain [19]. The optimum design of recombinant proteins which express well in bacteria and retain enzymatic activity is not trivial and to date only handful of them are produced. The AC7 C₂ described in this report is based on the published design [17] and as expected, behaved well during preparation. In order to include the previously identified ethanol responsive domain in the AC7 C_{1a} recombinant protein, we added 66 extra amino acid residues to the N-terminus of previously published optimized construct, which spans amino acid position 263–476 [17]. The expression and purification of this version of AC7 C_{1a} was poor (Fig. 1B). The recombinant AC9

Table 2
Regression associated parameters for butanol concentration response curves.

Fig. 7B				Fig. 7D			
	maximum value	Hill coefficient	EC50		maximum value	Hill coefficient	EC50
-Forskolin	2240 ± 56	1.80 ± 0.26	189 ± 10	-G α	312 ± 9	2.05 ± 0.36	335 ± 23
+Forskolin	9230 ± 332 ^a	1.88 ± 0.17	101 ± 6 ^a	+G α	7120 ± 177 ^a	2.15 ± 0.16	133 ± 5*

The parameters were obtained by regression analysis as described in Materials and Methods. Units used are pmol/mg protein* min for maximum values, μ M for EC50 (Fig. 7B), and mM for EC50 (Fig. 7D).

^a Significantly different from corresponding values shown directly above (*t*-test).

proteins were designed to be homologous to the corresponding AC7 recombinant proteins. Comparing to the previously published AC9 recombinant proteins [15], they are shorter, which may be contributed to better expression and purification. We tried to purify AC7sol, which we generated in our previous study [14]. However, we could not enrich the recombinant protein or AC activity by the His-tag affinity chromatography. This could be due to low level of expression and/or poor accessibility of His-tag on the protein.

The purified AC recombinant proteins were active even in the chimeric combination of the C_{1a} and C₂ domains, although the activity greatly varied. This is surprising since our previous study using a series of full length chimeric mutants showed that one of reciprocal chimeras of the C_{1a} and C₂ domains between AC7 and AC2 was not active [11]. Sequence wise, AC7 and AC2 are much more homologous than AC7 and AC9. This difference in compatibility of C_{1a} and C₂ domains could be due to the fact that one study used live mammalian cells expressing full length AC proteins and the other used recombinant proteins consisting of only cytoplasmic domains. Alternatively, the AC activity assay is much more sensitive compared to cAMP accumulation assay used in previous live cell based study.

When activated with MnCl₂, The activity of AC7 recombinant proteins (C_{1a}+C₂) was enhanced by ethanol. This result strongly suggests that ethanol directly interacts with AC protein to exhibits its effect. Our routine reaction mixture contains another mammalian enzyme, creatine phosphokinase, as a part of ATP regeneration system. We observed similar ethanol effect without the addition of this protein (data not shown). Thus, it appears that AC protein is the only protein necessary for ethanol to show its effect on AC activity. Under similar conditions, alcohol inhibited the activity of AC9 recombinant proteins. Thus, the ethanol effect has isoform specificity. Our previous study established that the isoform AC7 is most responsive to ethanol while AC9 has a weak response to ethanol [12]. The enhancing effect of ethanol on AC7 C_{1a}+AC7 C₂ was smaller than the effect observed using a full length AC7 expressed in live cells and the ethanol effect on AC9 C_{1a}+AC9 C₂ was reversed compared to the effect on a full length AC9, indicating that there are qualitative differences in alcohol effects on the AC recombinant proteins in the current study compared to the native form of AC. When ethanol effect was examined using chimeric combinations of C_{1a}+C₂, ethanol did not show significant effect. The results suggest that both C_{1a} and C₂ domains play some role in the action of ethanol, which is consistent with our previous study showing that both C_{1a} and C₂ domains contain an ethanol responsive domain [11]. The effects of butanol on AC7 C_{1a}+AC7 C₂ were examined in the presence of various combinations of forskolin, MnCl₂, and G α . The activation profile of AC7 C_{1a}+AC7 C₂ is very similar to that of AC7sol [14]. Also the direction of butanol effects (enhancement or inhibition) was similar. The only difference was observed in the presence of MnCl₂+G α : AC7 C_{1a}+AC7 C₂, inhibition; AC7sol, enhancement. It appears that the physical linkage of the C_{1a} and C₂ domains may cause this difference since this is the main difference between the two AC recombinant proteins. Regardless of the direction of the alcohol effects, all three forms of AC7, namely full length native form, AC7sol, and AC7 C_{1a}+AC7 C₂, have same alcohol cutoff point. This strongly suggests that the interaction of alcohol and AC7 protein is preserved in AC7

C_{1a}+AC7 C₂ and that observed differences in alcohol effects on the three forms of AC7 are due to the differences in their physical structures including presence/absence of membrane spanning domains and N-terminal domains and physical linkage of two cytoplasmic domains. However, we cannot rule out the possibility of unknown cellular factors involved in live cells since the magnitude of alcohol effect in live cells seems to be greater than that on bacteria expressed recombinant AC7.

Concentration response experiments shown in Fig. 7A and C indicated that butanol acted as a non-competitive antagonist for forskolin or G α in AC7 C_{1a}+AC7 C₂ activation, suggesting that alcohol interacts with an AC protein at a different site(s) from those binding to forskolin or G α and that alcohol allosterically modulates the activity of AC. Regardless of the presence of butanol, Hill coefficient for forskolin or GppNHP is very close to -1 (Table 1), which is consistent with the observations that one molecule of forskolin or G α interacts with one AC catalytic unit (C_{1a}+C₂) [19]. On the other hand, four concentration response curves for butanol shown in Figs. 7B and D have similar Hill coefficient (about 2, Table 2). This strongly suggests that alcohol has multiple binding/interaction sites on AC7 C_{1a}+AC7 C₂ and that alcohol binding/interaction to the AC catalytic unit has a positive cooperativity. Butanol increased EC50 for NKH 477, a water soluble forskolin analogue. Both forskolin and G α decreased EC50 for butanol. These results suggest that there are some interactions between the effect of alcohol and that of forskolin/G α .

In the current study, we used a high concentration of ethanol (200 mM) to accentuate the effect of ethanol on AC activity. However, lower concentrations of ethanol had clear effects as shown in Fig. 6. Our previous studies also showed that physiologically relevant concentrations of ethanol (less than 50 mM) significantly increased AC7 activity in HEK 293 cells [30]. We also showed that low concentrations of ethanol (less than 50 mM) enhanced the expression of a cAMP regulated reporter gene in AC7 transfected NIH 3T3 cells [10]. Concentration dependent curves shown in Figs. 7B and D indicate that EC50 for butanol ranges from 100 to 335 mM depending on the stimulants used to activate the AC7recombinant proteins (Table 2). Thus, 100 mM butanol used in Fig. 5 is not too high for biochemical characterization of alcohol effects. However, it should be noted that the most concentrations of alcohols used in this study are above physiological range.

5. Conclusion

Our study using purified recombinant AC proteins presented strong evidence suggesting that alcohol interacts directly with AC proteins to modulate its activity. These recombinant proteins provide useful research tools to investigate alcohol-protein interaction.

Competing interests

None.

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