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Effects of Alcohols on Recombinant Adenylyl Cyclase Type 7 Expressed in Bacteria

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Abstract

Background—Our previous studies showed that ethanol enhanced the activity of adenylyl cyclase (AC) in an isoform specific manner and that alcohol cutoff point of AC was isoform specific. Recently, we showed that 2,3-butanediol inhibited AC7 activity in a stereoisomer specific manner and that this inhibition was also AC isoform specific. These observations strongly suggest that a major target of alcohol action on cAMP signaling is AC. We hypothesized that alcohols exhibit their effect on AC activity by direct interaction with AC proteins. However, experimental systems employed in past studies such as intact cells and membrane preparations are too complex and do not allow us to unequivocally test this hypothesis. In attempt to bypass these complications of the membrane-bound AC, we decided to study the effect of alcohols on AC recombinant proteins expressed in bacteria.

Methods—A recombinant AC, designated as AC7sol, consisting of the C_{1a} and C₂ domains of the human AC type 7 (AC7) was designed and expressed in bacteria. The activity of AC7sol was examined using lysate prepared from bacteria expressing AC7sol.

Results—The activity of AC7sol was stimulated by manganese or by the α subunit of G protein that stimulates AC (G_{s α}). Forskolin by itself did not stimulate the activity of AC7sol. However, in the presence of activated G_{s α} , forskolin stimulated the activity of AC7sol. A series of *n*-alkanol including ethanol enhanced the manganese-stimulated activity of AC7sol. The alcohol cutoff point of AC7sol was pentanol. Ethanol and butanol increased V_{max} and K_M values of AC7sol.

Conclusion—These results are consistent with our hypothesis and suggest that the enhancing effect of alcohols on AC activity is due to the increase of turnover number of AC. The current study demonstrates for the first time that the effect of alcohols requires only the C_{1a} and C₂ domains of AC and no other domains of AC as well as no other mammalian proteins.

Keywords

Adenylyl cyclase; cAMP; recombinant protein; alcohol responsive domain

INTRODUCTION

Cyclic AMP signaling pathway has been postulated to play an important role in an animal's response to ethanol and in the predisposition to and development of alcoholism in humans. Ethanol alters the cAMP signaling in humans, animals, and model cell culture systems (Tabakoff and Hoffman, 1998). Therefore, we hypothesized that the alteration of cAMP signaling by ethanol results in part in pathophysiological consequences of ethanol

consumption and that elucidation of the mechanism by which ethanol modulates cAMP signaling may lead to the development of therapeutic agents and/or diagnostic tools.

The cAMP-generating system is a multicomponent system comprising membrane-bound AC, heterotrimeric G proteins, and G protein-coupled receptors. To date, nine different membrane-bound mammalian AC isoforms, type 1 to type 9, and one soluble AC isoform have been identified and characterized. Each isoform of AC has shown a distinct pattern of tissue distribution and regulation (Cooper, 2003; Sadana and Dessauer, 2009; Sunahara and Taussig, 2002). All membrane-bound ACs share a common structure including N-terminal tail (N) of various lengths, two cytoplasmic domains (C_1 and C_2) and two hydrophobic domains predicted to span the membrane six times each (M_1 and M_2). The C_1 and C_2 domains are divided into C_{1a} , C_{1b} , C_{2a} , and C_{2b} domains. The C_{1a} and C_{2a} domains form a heterodimeric catalytic core of the enzyme. We showed that ethanol enhances the activity of AC in an isoform specific manner and that AC7 is the most ethanol responsive isoform (Yoshimura and Tabakoff, 1995). Using a series of *n*-alkanols, we also showed that alcohol cutoff point of AC is isoform specific (Kou and Yoshimura, 2007). Recently, we have shown that 2,3-butanediol inhibits AC7 activity in a stereoisomer specific manner and that this inhibition is also AC isoform specific (Hasanuzzaman and Yoshimura, 2010). These observations strongly suggest that a major target of alcohol action on cAMP signaling is AC. By using a series of chimeric mutants between AC7 and other isoforms of AC, we showed that amino acid sequence differences in the N, M_1 , C_{1b} , and M_2 domains were not important for the enhancing effect of ethanol on AC (Yoshimura et al., 2006). Within the C_{1a} and C_2 domains, sequence difference in the N-terminal 28 residues of the C_{1a} domain and in the C-terminal 140 residues of the C_2 domain played an important role in the effect of ethanol on AC. We hypothesized that alcohols exhibit their effect on AC activity by direct interaction with AC proteins. However, experimental systems employed in past studies such as intact cells and membrane preparations are too complex and do not allow us to unequivocally test this hypothesis. The membrane-bound AC is not widely accessible due to its complicated structure and low expression level. Furthermore, the detergent-solubilized protein is labile and difficult to manipulate. In attempt to bypass these complications of the membrane-bound AC, we looked for an alternative experimental system.

Enzymatically active recombinant proteins derived from mammalian membrane-bound AC can be designed and expressed in bacteria. This was demonstrated for the first time by linking the C_{1a} domain of AC1 and the C_2 domain of AC2 with a short linker peptide (Tang and Gilman, 1995). This AC recombinant protein was soluble because of a lack of the membrane spanning domains, M_1 and M_2 . The activity of this recombinant AC was stimulated by activators for mammalian membrane-bound AC, such as forskolin and G_{sa} . Two forms of soluble recombinant AC derived from AC5 were also constructed by linking the C_1 or C_{1a} domain and the C_2 domain (Scholich et al., 1997). They were shown to respond to $G_{i\alpha 1}$ as well as forskolin and G_{sa} , and helped to locate the calcium binding site of AC5. The C_1 and C_2 or C_{1a} and C_{2a} domains can be expressed and purified separately and then mixed together to reconstitute catalytic activity (Whisnant et al., 1996; Yan et al., 1996). The development of these soluble recombinant proteins of membrane-bound AC greatly enhanced the understanding of the mechanisms by which the catalytic activity of membrane-bound AC is regulated (Sunahara and Taussig, 2002).

In order to examine whether alcohols enhance AC activity by directly interacting with AC molecules, we decided to study the effect of alcohols on AC recombinant proteins expressed in bacteria. In this paper, we describe the design and expression of a soluble form of the human AC7, designated as AC7sol. This protein has activity responding to manganese, G_{sa} , and forskolin. A series of *n*-alkanols including ethanol modulate the activity of this recombinant AC7.

MATERIALS AND METHODS

Materials

[³H] cAMP was from Moravsek Biochemicals (Brea, CA). [α -³²P]ATP was from Perkin Elmer (Boston, MA). Sodium dodecyl sulfate (SDS) was from Shelton Scientific (Shelton, CT). Phosphocreatine disodium salt hydrate and creatine phosphokinase, type III from bovine heart were obtained from Sigma (St Louis, MO). All other chemicals and reagents used were from Sigma-Aldrich, unless otherwise stated.

Construction of soluble form of AC7

A soluble form of AC7 recombinant protein, designated as AC7sol, was designed by joining the C_{1a} domain (amino acid residues 197 to 475) and the C₂ domain (amino acid residues 864 to 1080) with a 14-amino acid linker (Fig. 1). The N-terminal tail, two transmembrane domains (M₁ and M₂), and C_{1b} domain of AC7 were eliminated. This recombinant protein includes ethanol responsive domains previously identified (Yoshimura et al., 2006). Briefly, a 0.8-kb DNA fragment encoding the C_{1a} domain was amplified by PCR using pCMV-AC7 (Hellevuo et al., 1995) as a template, *PfuTurbo* DNA polymerase (Stratagene, La Jolla, CA), and two oligonucleotides (5'-ATCGGATCCACAAGCACCAAATGCAGGA and 5'-GAACCTCGAGGCCGCGTCCCCCTTGGGCC) as primers. The PCR product was digested with *Bam*HI and *Xho*I and ligated into pTrcHisC (Invitrogen, Carlsbad, CA) that was digested with the same enzymes. A 0.65-kb DNA fragment encoding the C₂ domain was amplified similarly using two oligonucleotides (5'-ATCGGATCCGACAAGTTAAACGAGGACTG and 5'-CAGAAGCTTCAGTTCAGCCCCAGCCCCCTGAAA) as primers. The PCR product was digested with *Eco*RI and *Hind*III and ligated into pTrcHisC containing the coding sequence of the C_{1a} domain that was digested with the same enzymes. A double stranded oligonucleotide encoding the 14-aa linker (forward; 5'-TCGAGTGCAGCAGGAGGAGCAGCAGGAGGAGCAGCG and reverse; 5'-AATTCGCTGCTCCTCCTGCTGCTCCTCCTGCTGCAC) was inserted into pTrcHisC containing the C_{1a} and C₂ domain coding sequences that was digested with *Xho*I and *Eco*RI. The resulting plasmid was designated as pTrcHisC-AC7sol. The coding sequence of AC7sol was confirmed by DNA sequencing analysis.

Expression of soluble AC7

The AC7 recombinant protein was expressed in an *E. coli* strain, TP2000, that carries a defective mutation in its AC gene (Tang and Gilman, 1995). Bacteria carrying pTrcHisC-AC7sol were grown at 25°C in T7 medium containing HP ampicillin (Geno Technology, Maryland Heights, MO). The expression of AC7sol was induced with 100 μ M isopropyl β -D-1-thiogalactopyranoside when A₆₀₀ reached 0.4, and bacterial cells were harvested 19 h after induction. Cells were suspended in ice-cold lysis buffer containing 20 mM Tris pH 8.0, 5 mM β -mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and Protease inhibitor cocktail set II (Calbiochem, La Jolla, CA), and lysate was prepared by breaking cells with a sonifier in the presence of 0.1 mg/mL lysozyme. The supernatant was collected after centrifugation at 150,000g for 30 min at 4 °C and stored under liquid nitrogen. The amount of protein was determined by BCA™ Protein Assay Kit-Reducing Agent Compatible (Thermo Scientific, Rockford, IL) using bovine serum albumin as a standard.

Expression and activation of G_{sa}

G_{sa} was expressed from the bacterial expression plasmid pQE60 (Qiagen, Valencia, CA) carrying the coding sequence of the bovine G_{sa} using the *E. coli* strain, TP2000, carrying pUBS520 that overexpresses *dnaY* gene product (Yan and Tang, 2002b). Bacteria growth

and lysate preparation were carried out following similar procedures as described for AC7sol, except induction with 30 μ M isopropyl β -D-1-thiogalactopyranoside. The lysate containing G_{sa} (100–125 μ g protein) was pre-activated by addition of the indicated concentration of Guanosine 5'-[β , γ -imido]triphosphate (GppNHp) in 20 mM Tris pH 8.0 and 3.5 mM MgCl₂ at 30°C for 30 min and put on ice approximately 10 min before adding into the reaction mixture.

Western blotting

Proteins in lysate (50 μ g) were separated by SDS-polyacrylamide gel electrophoresis (10% gel) and transferred to nitrocellulose membranes. AC7sol was detected by a polyclonal antibody specific to the C-terminal region of the C₂ domain of the human AC7 (Santa Cruz Biotechnology, Santa Cruz, CA). In addition, AC7sol was detected by the N-terminal polyhistidine tag using Universal His Western Blot Kit 2.0 (Clontech, Mountainview, CA). Signals were detected by ECL detection reagents (GE healthcare, Piscataway, NJ).

Enzymatic activity assay

The enzyme activity and alcohol response were studied by *in vitro* AC activity assay using [α -³²P] ATP as substrate (Smigel, 1986; Yoshimura and Cooper, 1992). Total 200 μ l reaction mixtures contained the lysate (100–125 μ g protein) containing AC7sol, 250 μ M ATP, 25 mM TrisHCl at pH 7.4, 100 μ M isobutylmethylxanthine, 10 mM phosphocreatine, 5U creatine phosphokinase, and [α -³²P]ATP (50–80 cpm/pmol). The concentrations of stimulants used are indicated in figure legends. Experiments shown in fig. 6 and 7 were carried out using different concentrations of ATP, as indicated in the legends. The reaction mixtures were prepared on ice before starting the assay by shifting temperature. The assays were performed at 30°C for 10–15 min. Under the assay conditions, the cAMP linearly increased over time up to 30 min (data not shown). The reaction was terminated by the addition of 200 μ l stop solution containing 2% SDS, 1 mM ATP and 1 mM cAMP. [³H] cAMP was added to each reaction before initiating column chromatography. Separation of [α -³²P] ATP and [α -³²P] cAMP was performed by sequential chromatography using Dowex 50 and neutral alumina columns and radioactive activity was quantified by a liquid scintillation counter (Salomon et al., 1974). The experiments were performed in triplicate and repeated at least twice to ascertain the consistency. Data in the figures are presented as mean $\pm$ S.D. of the representative experiments.

Data analysis

Data were plotted and analyzed using Sigma Plot (Systat Software, Point Richmond, CA). The statistical analyses were carried out using Sigma Stat (Systat Software, Point Richmond, CA).

RESULTS

Expression of AC7sol

Several recombinant proteins derived from mammalian AC that are enzymatically active have been designed and expressed in bacteria. Typically they are constructed by joining the two catalytic domains (C_{1a} and C_{2a}) with a short linker peptide or the two domains are expressed separately. Our previous study indicates that within the human AC7, the N-terminal region of the C_{1a} (position 197–224) and C-terminal region of the protein (position 941–1080) are important for the ethanol response of the enzyme (Yoshimura et al., 2006). Therefore, we constructed a gene encoding a recombinant AC7, designated as AC7sol, to include these regions as shown in Fig. 1. This protein contains the catalytic core of AC7 and regions of the protein important for ethanol response, and is devoid of the N-terminal, C_{1b},

M₁ and M₂ domains. The protein was expressed in TP2000 strain of *E. coli* as a single protein band with the mobility consistent with the expected molecular weight of 60.9 kDa by both N-terminal and C-terminal detection probes (Fig. 2). No significant amounts of smaller products were detected.

AC7 sol is enzymatically active

Under the assay condition employed, the bacterial lysate prepared from TP2000 containing empty vector (control) did not have any significant AC activity, while the lysate prepared from TP2000 expressing AC7sol showed much higher basal activity (Fig. 3). The activity of the control lysate did not respond to the addition of forskolin or manganese. On the other hand, the activity of the lysate containing AC7sol was increased in the presence of manganese. Addition of the lysate containing G_{sa} to the assay increased AC7sol activity, and addition of GppNHp further increased the AC7sol activity in a concentration dependent manner (Fig. 4a). Without added G_{sa} AC7sol activity did not change by GppNHp (data not shown). Addition of forskolin further increased AC7 sol activity about threefold at every concentration of GppNHp tested, while manganese shifted the GppNHp response curve upward in parallel. Forskolin by itself did not stimulate AC7sol activity under our assay condition (Fig. 3 and Fig. 4b), additionally, forskolin in the presence of manganese did not show much effect. Only at high concentrations (over 10 μ M) did forskolin increase AC7sol activity more than manganese alone. However, in the presence of activated G_{sa}, forskolin stimulated AC7sol activity in a clear concentration dependent manner: this is a sharp contrast compared to forskolin alone or forskolin plus manganese (Fig. 4b). Manganese stimulated AC7sol activity at higher concentrations (over 0.5 mM), while at lower concentrations, manganese did not have a significant effect on AC7sol activity (Fig. 4c). The manganese concentration response of AC7sol shifted from no effect to stimulation within a narrow concentration range. In the presence of activated G_{sa} or forskolin, AC7sol showed similar concentration response to manganese (Fig. 4c).

Effects of alcohols

Effects of short *n*-alkanols, including ethanol, on AC7sol activity were examined. AC7sol was activated with various combinations of manganese, G_{sa}, and forskolin (Fig. 5a and 5b). In the presence of manganese, all five *n*-alkanols enhanced the activity of AC7sol regardless of the addition of activated G_{sa} or forskolin. All *n*-alkanols tested had inhibitory effects on the activity of AC7sol stimulated by G_{sa} alone, however, addition of manganese reversed the effects of *n*-alkanols to enhancement. Forskolin alone did not increase the activity of AC7sol as shown in Fig. 3 and 4b. *n*-Alkanols did not have any effects on the activity of AC7sol in the presence of forskolin alone (data not shown).

The concentration-response curves of manganese-stimulated activity of AC7sol for four *n*-alkanols, ethanol, butanol, pentanol, and hexanol, were examined to determine alcohol cutoff point of AC7sol (Fig. 6). Concentration response curves progressively shifted to the left as the length of the *n*-alcohol increased from ethanol to pentanol. The response curves of pentanol and hexanol overlapped. There was no more left shift, indicating that alcohol cutoff point of AC7sol is pentanol. Pentanol is the same cutoff point observed for the wild type AC7 expressed in mammalian cells (Kou and Yoshimura, 2007).

Effects of ethanol and butanol on the kinetic parameters of AC7sol were examined (Fig 7a and b). V_{max} and K_M values were determined for AC7sol in the presence of 2.5 mM MnCl₂ with and without 200 mM ethanol or 100 mM butanol by nonlinear regression analysis using the following equation:

$$v = \frac{V_{\max}[ATP]}{K_M + [ATP]}$$

Similar K_M values were obtained from experiments using different batches of AC7sol preparation, however, $V_{\max}$ values were quite different (Table 1). Ethanol increased both $V_{\max}$ and K_M values of AC7sol stimulated by manganese. As a result the response curve in the presence of ethanol shifted upward and to the right at the same time, which made the response curves in the absence and presence of ethanol overlap at lower concentrations of ATP (<200 μ M). Butanol showed similar effects on the response curve. Both $V_{\max}$ and K_M values significantly increased in the presence of butanol.

DISCUSSION

In order to examine whether alcohols directly interact with AC to modulate the activity of the enzyme, we decided to design and express a recombinant AC7 in bacteria. The recombinant protein, AC7sol, includes two regions of AC7 previously identified as important for the enhancing effect of ethanol on AC7 (Yoshimura et al., 2006). The C-terminal boundary of the C_{1a} domain and the N-terminal boundary of the C_2 domain of AC7sol were determined based on the previous study in which the C_{1a} and C_2 domains of AC7 were designed and expressed separately (Yan and Tang, 2002a). This study addressed the importance of the design of recombinant AC to ensure proper folding and protection from proteolytic degradation and also mentioned that properly folded proteins form a uniform peak in an ion exchange chromatography. The authors claimed that these recombinant proteins are properly folded. In our study, AC7sol was constructed based on their design and structurally very similar. AC7sol remained as a soluble protein, was detected as a single polypeptide by both N-terminal and C-terminal specific probes demonstrating that no significant amount of proteolytic degradation products existed, and most importantly retained enzyme activity. Taken together, these results suggest that AC7sol is expressed as an intact form and is properly folded in the bacterial expression system. The main difference between AC7sol and previously prepared C_{1a} and C_2 recombinant proteins of AC7 (Yan and Tang, 2002a) are the addition of the N-terminal 66 residues of the C_{1a} domain and linkage of the two domains. The N-terminal region of the C_{1a} domain, which is important for ethanol effect on AC, was shown to be critical in the formation of an active conformation of the *Dictyostelium* AC (Parent et al., 2002). The 3D structure of this region is currently unknown.

TP2000 cells used for expression of AC7sol cannot produce cAMP, thus, the AC activity detected in the lysate prepared from TP2000 expressing AC7sol is derived solely from AC7sol as demonstrated in Fig. 3. This *E. coli* strain allows us to examine the activity of AC7sol without further purification, although purification is possible by utilizing the added hexahistidine tag on AC7sol. The activity of AC7sol responded to three stimulants commonly used for membrane-bound AC activation, namely G_{sa} , forskolin, and manganese. The response was concentration dependent for all three stimulants (Fig. 4a–c). The results indicate that AC7sol retains the enzymatic activity and basic regulatory characteristics of the original membrane-bound enzyme. However, there was one noticeable difference in the response of AC7sol compared to the previous study (Yan and Tang, 2002a): forskolin stimulated AC7sol only in the presence of activated G_{sa} and forskolin by itself or in combination with manganese had a very small effect. The previous study (Yan and Tang, 2002a) showed that forskolin by itself stimulated the activity of the mixture of separately expressed AC7 C_{1a} and AC7 C_2 , although the response was smaller compared to other soluble AC systems. The observed G_{sa} dependence of forskolin effect on AC7sol is most

likely due to the difference in protein structure between AC7sol and the mixture of separately expressed AC7 C_{1a} and AC7 C₂, which is described above. The previous study also showed that forskolin stimulated the activity of membrane-bound AC7 expressed in a baculovirus expression system. These observations suggest that the response of AC7sol may not be identical to that of the original membrane-bound enzyme.

A series of *n*-alkanols from ethanol to hexanol modulated the activity of AC7sol depending on the stimulants used. The *n*-alkanols themselves did not change the activity of AC7sol (data not shown). This is similar to our previous studies that employed a whole cell cAMP accumulation assay (Kou and Yoshimura, 2007). Our previous studies showed that in the whole cell cAMP accumulation assay, alcohols enhanced the activity of the native form of AC7 stimulated with G_{sα} (Hasanuzzaman and Yoshimura, 2010; Kou and Yoshimura, 2007). However, in the current study, the G_{sα}-stimulated activity of AC7sol was decreased by addition of *n*-alkanols including ethanol. To observe the enhancement of the G_{sα}-stimulated activity of AC7sol, addition of millimolar concentration of manganese was required. If manganese was present, the activity of AC7sol was increased by *n*-alkanols regardless of the presence of either G_{sα} or forskolin (Fig. 5a). The intracellular concentration of manganese was determined to be less than 1 μM for mammalian cells (Milne et al., 1990). Manganese at 1 μM had no effect on the activity of AC7sol or on alcohol effects on the G_{sα}-stimulated activity of AC7sol (data not shown). This suggests that the enhancing effects of alcohols on the G_{sα}-stimulated activity of native AC7 observed in mammalian cells (Kou and Yoshimura, 2007) are not dependent on manganese present in the cells.

This manganese-dependence of stimulatory alcohol effects seems to be unique to AC7sol and likely resulted from the design of AC7sol. Our previous study (Yoshimura et al., 2006) indicated that sequence differences in other domains (N, M₁, M₂, and C_{1b}) are not important for ethanol effect on AC. However, this does not mean that these domains are not required for the effect of ethanol to manifest. For example, the sequence in the M₁ and M₂ domains are very diverse among AC isoforms and exchange of them between AC isoforms did not affect the degree of ethanol effect. However, the very fact that the M₁ and M₂ domains bring the catalytic domains (C_{1a} and C₂) close to plasma membrane may be important for alcohol effect. Or the M₁ and M₂ domains may align the catalytic domains to a conformation necessary for ethanol effect. It is also possible that unknown posttranslational modification of the native AC7 makes the native enzyme manganese independent in terms of enhancement by alcohols. Alternatively, the linkage of the C_{1a} and C₂ domains with a short peptide sequence could be the cause of the manganese-dependence of stimulatory alcohol effects just as this could be the cause of the G_{sα} dependence of forskolin effect on AC7sol as discussed above. In addition, the possibility that unknown bacterial proteins cause the change cannot be ruled out since AC7sol was in bacterial lysate.

Ethanol concentration response of AC7sol shown in Fig. 6 is comparable to that of native AC7 examined in mammalian cell culture in previous studies (Yoshimura et al., 2006; Yoshimura and Tabakoff, 1999) suggesting that AC7sol retains ethanol responsiveness of the native AC7. However, whether isoform specificity of ethanol effect observed for native AC isoforms (Yoshimura and Tabakoff, 1995) is maintained with AC7sol needs to be determined. For this reason, similar recombinant AC proteins derived from other isoforms need to be prepared in future studies. The observed effect of physiologically relevant concentrations of ethanol (25 and 50 mM) on AC7sol activity may appear to be small. However, the change in AC activity could generate a substantially larger change in cAMP in mammalian cells when phosphodiesterase is active (Yoshimura and Tabakoff, 1999).

The alcohol cutoff point was defined as *n*-alkanol with a certain carbon chain length beyond which there is no further increase in the potency of an ascending series of *n*-alkanols (Franks

and Lieb, 1985). Our previous study indicates that the alcohol cutoff point of membrane-bound AC is isoform specific. Thus, each AC isoform has a unique alcohol cutoff point. The cutoff points of AC6, AC7, and AC9 were determined to be pentanol, butanol, and beyond decanol, respectively (Kou and Yoshimura, 2007). The alcohol cutoff point of AC7sol was determined to be pentanol in the current study. This is in agreement with that of the native AC7 determined in the whole cell cAMP accumulation assay. The results indicate that AC7sol retains alcohol responsiveness and regulatory properties of the original AC7 for the most part. Therefore, the soluble form of AC7 recombinant protein expressed in bacteria provides a valuable model system to study alcohol effects on the activity of AC.

The current study has demonstrated for the first time that the C_{1a} and C₂ domains are sufficient to exhibit the enhancing effect of alcohol on the activity of AC. This is in agreement with our previous study showing that the amino acid sequence differences in the N, M₁, C_{1b}, and M₂ domains were not important for enhancing effect of ethanol on AC (Yoshimura et al., 2006) and goes one step further to suggest that these domains are not required for the effect of alcohols. Since AC7sol was expressed in bacteria, our results indicate that no other mammalian proteins are necessary for the observed alcohol effect on AC7sol and strongly suggest that alcohols directly interact with the C_{1a} and/or C₂ domain to exhibit the observed effects. Whether alcohols interact with a specific binding site as suggested in the case of ligand-gated channels including GABA_A receptor and glycine receptor (Mihic et al., 1997) needs to be determined. We showed in a previous study that ethanol-induced phosphorylation of AC7 may play a role in the enhancement of the activity of AC7 by ethanol (Nelson et al., 2003). However, the results of the current study suggest that the primary effect of alcohols is through direct interaction with AC and that the ethanol-induced post-translational modification of AC may not be a primary cause of the alcohol effect on AC. Both ethanol and butanol increased V_{max} and K_M values of AC7sol. 10 mM hexanol also showed a tendency to increase V_{max} and K_M values of AC7sol but the changes were not significant (data not shown). It appears that an increase in V_{max} and K_M values is a common characteristic of action of straight chain alcohols on AC function.

In summary, the current study demonstrates for the first time that the enhancing effect of alcohols on the activity of AC can be detected using a bacterially expressed recombinant AC7 consisting of only the C_{1a} and C₂ domains. The soluble form of AC recombinant proteins presents a valuable model system to study the mechanism by which alcohols modulate the activity of AC.

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Fig. 1.

Schematic representation of AC7sol. The native form of AC7 structure (top) and AC7sol, a soluble form of AC7 (bottom), are illustrated. AC7sol was constructed by fusing the C_{1a} and C₂ domains with a 14-amino acid linker. The numbers indicate the positions of the residues in the human AC7. 6xHis indicates a hexahistidine tag.

Fig. 2.

Detection of AC7sol in lysate. The lysate derived from bacteria carrying empty vector (C) and that derived from bacteria carrying pTrcHisC-AC7sol (AC7) were analyzed by Western blotting as described in Materials and Methods. An antiAC7 antibody was used for blot 1 and Universal His Western Blot Kit 2.0 was used for blot 2.

Fig. 3.

Comparison of AC activity in lysate. Representative AC activity of the lysate derived from bacteria carrying empty vector (Control) and that derived from bacteria carrying pTrcHisC-AC7sol (AC7) are shown. The activity was detected without stimulants (Basal) and in the presence of 10 μ M forskolin (Fsk) or 2.5 mM MnCl_2 (Mn^{2+}) as described in Materials and Methods. The experiments were carried out at 30°C for 15 min.

Fig. 4.

Concentration responses of AC7sol to various stimulants. a) GppNHp concentration response curves. $G_{s\alpha}$ was pre-incubated with various concentrations of GppNHp. The AC activity assays were performed in the presence of activated $G_{s\alpha}$ alone (Gs) or with addition of either 1 mM $MnCl_2$ (Gs + Mn^{2+}) or 10 μ M forskolin (Gs + Fsk). b) Forskolin concentration response curves. The AC activity assays were performed at various concentrations of forskolin in the presence of forskolin alone (Fsk) or with addition of either $G_{s\alpha}$ activated with 10 μ M GppNHp (Fsk + Gs) or 1 mM $MnCl_2$ (Fsk + Mn^{2+}). c) Manganese concentration response curves. The AC activity assays were performed in the presence of various concentrations of $MnCl_2$ alone (Mn^{2+}) or with addition of either $G_{s\alpha}$ activated with 10 μ M GppNHp (Mn^{2+} + Gs) or 10 μ M forskolin (Mn^{2+} + Fsk). The experiments were carried out at 30°C for 10 min. The three plots in a) and one of the plots in b) (Gs + Fsk) were prepared by nonlinear regression using the following equation:

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

Fig. 5.

Effects of alcohols on AC7sol. The effects of a series of *n*-alkanols on AC7sol were examined. *n*-alkanols and their concentrations used are indicated in the graphs. Percent stimulation by addition of each *n*-alcohol compared to the activity without alcohol is displayed. a) AC activity assays were carried out in the presence of 1 mM $MnCl_2$ alone (Mn^{2+}) or in addition of either G_{sa} activated with 10 μ M GppHNP ($Mn^{2+} + G_s$) or 10 μ M forskolin ($Mn^{2+} + Fsk$). b) AC activity assays were carried out in the presence of G_{sa} activated with 10 μ M GppHNP alone (G_s) or in addition of either 1 mM $MnCl_2$ ($G_s + Mn^{2+}$) or 10 μ M forskolin ($G_s + Fsk$). The experiments were carried out at 30°C for 10 min.

Fig. 6.

Alcohol cutoff of AC7sol. The effect of indicated *n*-alkanols on the activity of AC7sol was measured at various concentrations in the presence of 1 mM ATP and 2.5 mM MnCl₂. The results are displayed as percent stimulation by alcohol plus manganese over stimulation by manganese alone. The experiments were carried out at 30°C for 15 min.

Fig. 7.

Determination of V_{max} and K_M for AC7 sol. The activity of AC7sol was determined in the presence of 2.5 mM $MnCl_2$ using various concentrations of ATP. a) The activity was measured in the absence or presence of 200 mM ethanol as indicated. b) The activity was measured in the absence or presence of 100 mM butanol as indicated. Different batches of protein lysates were used for each experiment. The experiments were carried out at 30°C for 15 min.

Table 1

Kinetic parameters of AC7sol

	K_M (μM) ^a	V_{max} (pmole/mg/min) ^a
Exp 1 Control	334±42	188±10
Exp 1 +Ethanol	579±89 [*]	274±21 ^{**}
Exp 2 Control	407±43	360±17
Exp 2 +Butanol	537±35 [*]	678±21 ^{***}

^aValues are expressed as mean±S.E.*, **, *** Values are significantly different from the matching control values by *t*-test. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$.