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Real-time monitoring of intracellular cAMP during acute ethanol exposure

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Abstract

Background—In previous studies we have shown that ethanol enhances the activity of Gs-stimulated membrane-bound adenylyl cyclase (AC). The effect is AC isoform specific and the type 7 AC (AC7) is most responsive to ethanol. In this study, we employed a fluorescence resonance energy transfer (FRET) based cAMP sensor, Epac1-camps, to examine real-time temporal dynamics of ethanol effects on cAMP concentrations. To our knowledge, this is the first report on real-time detection of the ethanol effect on intracellular cAMP.

Methods—Hela cells were transfected with Epac1-camps, dopamine D_{1A} receptor, and one isoform of AC (AC7 or AC3). Fluorescent images were captured using a specific filter set for cyan fluorescent protein (CFP), yellow fluorescent protein (YFP), and FRET, respectively and FRET intensity was calculated on a pixel-by-pixel basis to examine changes in cAMP.

Results—During 2-minute stimulation with dopamine (DA), the cytoplasmic cAMP level quickly increased, then decreased to a plateau, where the cAMP level was higher than the level prior to stimulation with DA. Ethanol concentration dependently increased cytoplasmic cAMP in cells transfected with AC7, while ethanol did not have effect on cells transfected with AC3. Similar trends were observed for cAMP at the plasma membrane and in the nucleus during 2-minute stimulation with DA. Unexpectedly, when cells expressing AC7 were stimulated with DA or other Gs protein-coupled receptor's ligand plus ethanol for 5 seconds, ethanol reduced cAMP concentration.

Conclusion—These results suggest that ethanol has two opposing effects on the cAMP generating system in an AC isoform specific manner, the enhancing effect on AC activity and the short lived inhibitory effect. Thus, ethanol may have a different effect on cAMP depending on not only AC isoform but also the duration of exposure.

Keywords

Adenylyl cyclase; Ethanol; cAMP; FRET; Real-time measurement

INTRODUCTION

Cyclic AMP (cAMP) is a ubiquitous second messenger molecule that plays important regulatory roles in diverse biological processes including learning and memory. The common cAMP generating system is a membrane-bound system, which includes AC, heterotrimeric G proteins, and G protein-coupled receptors (GPCR). The activity of membrane-bound AC is regulated by a variety of extracellular molecules such as hormones,

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neurotransmitters, and odorants. Numerous clinical studies have suggested that AC and the cAMP signaling system play an important role in the predisposition to and development of alcoholism (Diamond et al., 1987; Lex et al., 1993; Menninger et al., 1998; Parsian et al., 1996; Saito et al., 1994; Tabakoff et al., 1988; Waltman et al., 1993). Compared to control subjects, alcoholic individuals have lower AC activity in their lymphocytes and platelets. Low platelet AC activity is characteristic of individuals with a family history positive for alcoholism, whether or not the subjects themselves are diagnosed as alcoholics (Lex et al., 1993; Menninger et al., 2000; Ratsma et al., 1999). cAMP signaling pathways play an important role in an animal's response to ethanol as well. For example, type 5 AC (AC5) knockout mice have a reduced sensitivity to the sedative effects of ethanol and increased ethanol consumption (Kim et al., 2011). In contrast, type 1 AC (AC1) knockout mice have an increased sensitivity to the sedative effects of ethanol and type 8 AC (AC8) knockout mice show decreased ethanol consumption (Maas Jr et al., 2005). In *Drosophila*, inactivation of *rutabaga*, an AC gene, increases the fly's sensitivity to ethanol-induced sedation (Moore et al., 1998).

In Mammals, nine membrane-bound 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). Isoform specific changes in the expression levels have been reported for several pathological conditions. For example, alterations of specific AC isoforms have been found in brains of alcoholics (Hashimoto et al., 1998; Yamamoto et al., 2001), heroin addicts (Shichinohe et al., 2001), and Alzheimer's disease patients (Yamamoto et al., 1996).

Ethanol alters the cAMP signaling pathways in brain and other tissues in animal models as well as in model cell culture systems. In general, acute exposure to ethanol enhances receptor-stimulated and/or G protein-stimulated AC activity, while chronic exposure to ethanol often decreases AC activity (Tabakoff and Hoffman, 1998). Therefore, we assumed that the alteration of cAMP signaling by ethanol is, in part, responsible for the 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. We showed that ethanol enhances the activity of AC in an AC isoform specific manner and that AC7 is the most ethanol-responsive AC isoform (Yoshimura and Tabakoff, 1995). Previous research indicates that the activity of AC7 can be significantly potentiated by 10 to 20 mM ethanol (Yoshimura and Tabakoff, 1999); this range of ethanol concentrations can easily be attained in the blood by consuming alcoholic beverages. Studies using a series of straight chain alcohols indicated that the alcohol cutoff effect for *n*-alkanol potentiation of AC activity is AC isoform specific (Kou and Yoshimura, 2007) and that 2,3-butanediol inhibits AC7 activity in a stereoisomer specific manner as well as in an AC isoform specific manner (Hasanuzzaman and Yoshimura, 2010). Based on these observations, we hypothesized that within the cAMP – generating system, AC is a main target of alcohols including ethanol and that alcohols interact directly with AC molecules. We have identified regions of the AC7 protein that are important for ethanol's enhancing effect using a series of chimeric mutants (Yoshimura et al., 2006). Using a bacterial expression system, we have shown that the activity of a recombinant AC7 lacking membrane-spanning domains can be enhanced by alcohols including ethanol in the absence of other mammalian proteins (Dokphrom et al., 2011), strengthening the hypothesis.

The recent development of cAMP sensors that utilize fluorescence resonance energy transfer (FRET) between two variants of green fluorescent protein (GFP) enables us to determine cAMP metabolism in real-time in living cells (Nikolaev and Lohse, 2006). These sensors

can provide intracellular cAMP levels with unprecedented spatial and temporal resolutions. By utilizing these sensors it became possible to monitor dynamics of cAMP synthesis and hydrolysis, which has revealed the existence of different cAMP compartments in cells (Berrera et al., 2008; Iancu et al., 2008). To our knowledge, the real-time monitoring of the effects of ethanol on intracellular cAMP metabolism has not been reported. In this report, we examine the effect of ethanol on cAMP levels in cells expressing either AC7, the most ethanol responsive isoform, or AC3, the least ethanol responsive isoform, using a cAMP sensor, Epac1-camps (Nikolaev et al., 2004), directed to three different compartments: cytoplasm, nucleus, and plasma membrane. The results provide valuable insight regarding the molecular mechanisms of the ethanol effect on cAMP signaling.

MATERIALS AND METHODS

Materials

Minimal essential medium (MEM), and penicillin–streptomycin–neomycin antibiotic mixture were obtained from Invitrogen (Grand Island, NY). Fetal bovine serum was provided by Hyclone (Logan, UT). Dopamine (DA), 2-p-(2-carboxyethyl) phenethyl amino-5'-N-ethy-carboxamidoadenosine (CGS21680), Amthamine dihydrobromide, isoproterenol, 3-isobutyl-1-methylxanthine (IBMX), and trichloroacetic acid were all purchased from Sigma-Aldrich (St Louis, MO). [32 P]ATP was purchased from Perkin Elmer (Boston, MA). [14 C]cAMP and [3 H]adenine were obtained from Moravsek Biochemicals (Brea, CA). Plasmids containing the coding sequences of AC7, AC3, Epac1-camps, and the D_{1A} dopamine receptor (DRD1A) were described previously (Dearry et al., 1990; Nikolaev et al., 2004; Yoshimura et al., 2006). The pCMV-XL, containing encoding sequences of human adenosine A_{2A} receptor (ADORA2A), adrenergic α_2 receptor (ADRB2), and histamine H₂ (HRH2) under CMV promoter were purchased from Origene (Rockville, MD).

Construction of Epac1-camps targeted to subcellular compartments

Single polypeptide sensor for measuring cAMP, Epac1-camps (Nikolaev et al., 2004), was used as the template to generate Epac1-camps targeted to subcellular compartments as described previously (Allen and Zhang, 2006; DiPilato et al., 2004). Briefly, for plasma membrane targeting of Epac1-camps, the plasma membrane-targeting sequence of small GTPase K-ras4B, KKKKKSKTKCVIM, was inserted at the C-terminus. For cytoplasmic expression, nuclear export signal (NES) of the HIV-1 Rev protein, LPPLERLTL, was inserted to the C-terminus. For nuclear targeting, the nuclear localization signal (NLS), PKKKRKVEDA, was inserted to the C-terminus. The addition of coding sequences for the respective tag peptides to Epac1-camps gene were carried out by PCR-based mutagenesis using specific primers. The mutations were confirmed by DNA sequencing. Correct targeting of the Epac1-camps was confirmed by fluorescent microscopy (Fig. 1).

Cell culture and transfection

Hela cells were obtained from American Type Culture Collection (Manassas, VA). The cells were maintained in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. Cells were cultured on polyethyleneimine coated glass coverslips in a 12 well plate containing 1 ml of MEM with 10% fetal bovine serum, penicillin (50 µg/ml), streptomycin (50 µg/ml), and neomycin (100 µg/ml). Transfection was performed using Lipofectamine 2000 following manufacturer's instructions (Invitrogen, Carlsbad, CA) with genes encoding Epac1-camps, one of GPCRs (DRD1A, HRH2, ADORA2A, or ADRB2), and one isoform of AC (AC3 or AC7). After approximately 24 hours, Time-lapse FRET imaging of living cells was carried out.

Live cell fluorescence imaging

A 15mm coverslip with transfected Hela cells was assembled in a perfusion chamber (87 μ l/mm depth with ~10ml/min flow rate) attached to an eight channel perfusion valve control system (Warner Instruments, Hamden, CT) and placed on a microscope stage. The fluorescent imaging workstation consists of an inverted microscope (Olympus IX81), a 60X oil immersion lens, a cooled charge-coupled device camera (Hamamatsu ORCA-AG), dual filter wheels, and a xenon light source, all controlled by a computer with SlideBook 5.1 software (Intelligent Imaging Innovation, Denver, CO). Filter sets for image capture were YFP (excitation, 500/20 nm; emission, 535/30 nm), CFP (excitation, 430/24 nm; emission, 470/24 nm), and FRET (excitation, 430/24 nm; emission, 535/30 nm). A dichroic mirror (T89002bs, Chroma, Bellows Falls, VT) was used. Cells were constantly perfused with KRH buffer (NaCl 129mM, NaHCO₃ 5mM, KCl 4.8mM, KH₂PO₄ 1.2mM, CaCl₂ 1mM, glucose 2.8mM, HEPES 10mM, MgCl₂ 1.2mM, pH 7.2) at room temperature. Drugs were added for the durations indicated in the figures, through the perfusion system. Three fluorescent images at each time point were captured using a specific filter set for CFP, YFP, and FRET, respectively. Binning 2X2 modes and 100–500 milliseconds integration time were used. Images were captured every 3 seconds for the duration of time-lapse experiments.

FRET Measurements

The method of sensitized FRET measurement was described previously (Sorkin et al., 2000). The background images were subtracted from the raw images before FRET calculations. The corrected FRET (cFRET) was calculated on a pixel-by-pixel basis for the entire image using Equation1: $cFRET = FRET - (0.9088 \times CFP) - (0.025 \times YFP)$, where FRET, CFP, and YFP correspond to background-subtracted images of cells expressing Epac1-camps acquired through the FRET, CFP, and YFP channels, respectively. The values 0.9088 and 0.025 are the fractions of bleed-through of CFP and YFP fluorescence, respectively, through the FRET filter channel. Normalized FRET (nFRET) values for individual cellular compartments were calculated according to Equation2: $nFRET = [cFRET] / ([YFP] \times [CFP])$, where [cFRET], [CFP], and [YFP] are the mean emission intensities of cFRET, CFP, and YFP fluorescence in the selected subregion. The nFRET values were normalized by dividing by the mean of nFRET values of the first 20 time points for plotting.

cAMP accumulation assay

The amounts of cAMP in the transfected Hela 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 μ Ci/ml of [2,8-³H]adenine. To determine the effect of ethanol, the cells were treated with various concentrations of ethanol together with 1 μ M DA for 30 seconds at 37°C. The reaction was terminated by adding 50 μ 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 by liquid scintillation spectrometer. [-³²P]ATP and [8-¹⁴C]cAMP were added as internal standards to monitor the recovery of ATP and cAMP through column chromatography.

Statistics

The experiments were repeated at least three times. For FRET imaging, the values are expressed as mean $\pm$ standard error of mean (SEM) of the results obtained from different cells. Student's *t*-test, one-way ANOVA, or two-way repeated-measures ANOVA was used to evaluate differences in values as indicated in figure legends. After ANOVA, pairwise comparison was carried out by Holm-Sidak method.

RESULTS

Ethanol effect is concentration dependent and AC isoform specific

Cells expressing Epac1-camps, dopamine D_{1A} receptor, and AC7 or AC3 were stimulated with DA $\pm$ ethanol for 2 minutes to monitor real-time change in cytoplasmic cAMP. Normalized nFRET values were plotted over time as an indicator of cAMP. In cells expressing AC7, nFRET rapidly decreased after addition of 0.2 μ M DA (Fig. 2A). The decrease was detected at the first FRET measurement (<3 seconds) after addition of DA. The value of nFRET reached its minimum ~ 20 seconds after addition of DA and increased to a plateau after ~ 60 seconds and remained constant during the rest of the DA treatment period (~ 60 seconds). The nFRET values at the plateau were significantly lower than the initial values before DA addition. Thus, cytoplasmic cAMP rapidly increased to its peak value and then decreased to a value which was higher than the initial concentration, and stayed during the recording period. The addition of ethanol (100 or 200 mM) together with DA significantly increased cytoplasmic cAMP in a concentration dependent manner. The change of cAMP concentration followed a similar time course as stimulation with DA alone but time to reach to the peak concentration and plateau was longer in the presence of ethanol. Ethanol at 25 mM and 50 mM did not increase cAMP reproducibly over DA alone (data not shown). However, cAMP accumulation assay using a population of cells clearly showed enhancement of cAMP production by ethanol at 25 and 50 mM (Fig. 2B). Addition of ethanol alone (200 mM) did not cause any changes in nFRET values (data not shown) indicating that FRET signal of Epac1-camps is not affected by ethanol. Cells expressing AC3 showed similar cytoplasmic cAMP change during 2-minute stimulation with DA, however, ethanol did not have any significant effects on cAMP at 100 and 200 mM (Fig. 3).

Ethanol effect on cAMP at plasma membrane and in nucleus

cAMP level at the plasma membrane and in the nucleus of the cells expressing AC7 was examined during 2-minute stimulation with DA (Fig. 4A and B). Both at the plasma membrane and in the nucleus, cAMP level increased rapidly to a peak concentration then decreased to a plateau. The time course of the cAMP change was similar to that in the cytoplasm. When ethanol was added together with DA, cAMP in the nucleus followed a similar increase to that of cAMP in cytoplasm. However, at the plasma membrane higher levels of cAMP in the presence of ethanol were short lived (~ 30 seconds). cAMP decreased to the plateau similar to the one observed for cells treated with DA alone. cAMP level at the plasma membrane and in the nucleus of the cells expressing AC3 followed a similar time course to those of cells expressing AC7 during 2-minute stimulation with DA. Ethanol had no significant effects on the time course or amount of cAMP level at the peak or at the plateau in cells expressing AC3 (Fig. 4C and D).

Effect of long exposure to ethanol on cAMP in cells expressing AC7

To examine the effects of long exposure to ethanol, cells were incubated in the presence of 50 mM ethanol for 24 hours after transfection and cytoplasmic cAMP level during 2-minute stimulation with DA was recorded (Fig. 5). The time course of cAMP change was similar to that of the cells that were not exposed to ethanol during 24 hours preceding the recording (naïve cells). In addition, the enhancing effect of ethanol added together with DA was also similar to that observed in the naïve cells. The results indicated that under the experimental condition, long exposure to ethanol (up to 24 hours) had little effects on the cAMP generation system and on the enhancing effect of ethanol. Similar results were observed with 4-hour exposure with 50 mM ethanol (data not shown).

Effect of ethanol on very short stimulation with DA

Cytoplasmic cAMP level of the cells expressing AC7 was examined when DA was added for 5 seconds (Fig. 6A and B). Based on the volume of the perfusion chamber (87 μ l/mm depth) and flow speed (~10 ml/min), it was expected that the liquid in the chamber was replaced within a few seconds. cAMP rapidly increased right after addition of DA, kept rising after removal of DA and reached its peak ~18 seconds after the start of DA addition (Fig. 6A). When cells were treated with DA plus ethanol for 5 seconds, cytoplasmic cAMP increase was significantly smaller than that of cells treated with DA alone. When a similar experiment was carried out in the presence of a non-specific phosphodiesterase (PDE) inhibitor, IBMX, ethanol diminished DA-stimulated cAMP production (Fig. 6B), demonstrating that the observed reduction of cAMP in the presence of ethanol was due to reduced activity of AC7 not due to enhanced PDE activity. The results indicated that very short exposure to ethanol (5 seconds) during stimulation with DA decreased the activity of AC7 to the contrary of the observation with longer exposures (>30 seconds) shown in Fig. 2 and previous studies using radiochemical assay with a population of cells.

Ethanol effects are not specific to D_{1A} but common for GPCRs

When cells expressing either adenosine A_{2A}, adrenergic α_2 , or histamine H₂ receptors together with AC7 and Epac1-camps targeted to cytoplasm were stimulated with respective agonist and ethanol for 2 minutes, ethanol significantly increased cytoplasmic cAMP concentration (Fig. 7A–C). However, when cells were stimulated with the respective agonist and ethanol for 5 seconds, ethanol either reduced cytoplasmic cAMP or had no significant effect compared to that observed with the ligand alone (Fig. 7D–F). Thus, the dichotomy of ethanol effect appeared to be common for Gs-coupled receptors.

Inhibitory effect of ethanol is transient and isoform specific

In order to examine whether the observed inhibition by ethanol is due to the short exposure to ethanol or due to the short duration of stimulation with a GPCR agonist, ethanol exposure time was changed while stimulation with DA was kept as 5 seconds (Fig. 8). Exposure to ethanol for 2 minutes prior to 5-second stimulation with DA increased cytoplasmic cAMP in cells expressing AC7 compared to cells with no ethanol exposure, while application of DA and ethanol for 5 seconds decreased cAMP (Fig. 8A). Thus, the inhibition is due to the short ethanol exposure and not due to the short stimulation with DA. When cells expressing AC3 were employed in a similar experiment (Fig. 8B) ethanol did not have any significant effect on cytoplasmic cAMP regardless of the duration of exposure.

DISCUSSION

We established that the activity of AC, which generates cAMP, is modified by ethanol in an isoform specific manner and that intracellular levels of cAMP are modulated by pharmacologically relevant concentrations of ethanol in cells expressing AC7, as determined by cAMP accumulation assay (Yoshimura and Tabakoff, 1995; 1999). Emerging studies indicate that GPCR-stimulated cAMP signals are highly dynamic and complex. To gain insight into this signaling, monitoring intracellular cAMP with high spatiotemporal resolution is necessary. In this study, we have used a FRET-based cAMP sensor, Epac1-camps, permitting us to investigate the effect of ethanol on cAMP levels in HeLa cells with high spatiotemporal resolution. Epac1-camps contains a single cAMP binding domain of Epac1 between the two fluorescent protein moieties and has an EC₅₀ Value of 2.4 μ M, which is suitable to measure cAMP within the physiological range of concentrations. In addition, Epac1-camps has a larger signal amplitude and higher response speed compared to other cAMP sensors (Nikolaev et al., 2004; Nikolaev and Lohse, 2006).

Our initial observations with cells expressing AC7 or AC3 together with the D_{1A} dopamine receptor (Fig. 2 and 3) are similar to the previous study showing ethanol effects on cAMP in an ethanol concentration and an AC isoform dependent manner. However, FRET-based analysis did not show significant effect of ethanol at 25 and 50 mM concentrations on AC7 expressing cells while cAMP accumulation assay with population of cells clearly showed the effects (Fig. 2B). This result could be attributed to lower sensitivity of Epac1-camps at lower concentrations (submicromolar) of cAMP under the experimental condition or lower statistical power since the number of cells examined was limited. It is also possible that the selection of cells for recording created unexpected bias since cells with very bright spots inside or very dark signals were avoided from recording.

Time course of cAMP change during 2-minute stimulation with a GPCR agonist, regardless of GPCR used, displayed a peak at ~20 seconds and a plateau within 60 seconds. This kind of time course (peak & plateau) for cAMP with longer duration to reach the peak were reported and attributed to GPCR desensitization (Ponsioen et al., 2004). It is also possible that delayed onset of PDE activity plays some role in the observed time course. The time courses we presented in this report are rapid but still appear to be within the time course of GPCR desensitization since this could occur within seconds of stimulation (Ferguson, 2001). However, some reports show a sustained high level of cAMP while stimulants are present (DiPilato et al., 2004; Nikolaev et al., 2004; Tateyama and Kubo, 2006). These differences in cAMP changes could be due to cell types and GPCR employed. Also, some observations could be erroneous due to the employed cAMP sensor molecule. For example, when thrombin induced cAMP was examined in endothelial cells, PKA-based sensor, which releases an active PKA catalytic subunit upon cAMP binding displayed a very different cAMP time course compared to Epac1-camp (Werthmann et al., 2011). PKA overexpressed as a sensor could activate PDEs and alter cAMP time course. Since we used Epac1-camps, which is depleted of any functional domains other than a cAMP binding site, cAMP time courses shown here represent actual changes in cAMP without interference by the sensor.

There was little difference in the time course of cAMP changes in the plasma membrane, cytoplasm, and nucleus. Considering the diffusion coefficient of cAMP ($487 \pm 23 \mu\text{m}^2/\text{s}$) (Nikolaev et al., 2004) and the size of Hela cells it is not surprising not to detect time course differences between the three subcellular compartments under the experimental condition. Also, little differences in cAMP concentrations were observed in the three compartments. This is contrary to previous reports regarding cAMP changes in HEK293 cells (DiPilato et al., 2004; Terrin et al., 2006), which showed slower increase and lower concentrations of cAMP in cytoplasm compared to plasma membrane. This difference could be due to the diversities between the two cell types in quantity and distribution of PDEs, which are important in shaping compartmentalization of cAMP (Terrin et al., 2006). Ethanol increased cAMP in all three compartments in cells expressing AC7 (Fig. 2A, Fig. 4A and 4B) but caused little change in time course of cAMP. If anything, time to reach peak concentration delayed slightly. One noticeable difference among the three compartments was that cAMP level at plateau at the plasma membrane was similar with or without ethanol, while in the other two compartments, cAMP level at plateau was significantly higher with ethanol suggesting that the regulation of cAMP level at the plasma membrane is different from the other two compartments. This could be due to the difference in isoform(s) of PDE residing in these compartments.

24-hour ethanol exposure of Hela cells expressing AC7 did not alter time course or ethanol effect on cAMP during 2-minute stimulation (Fig. 5) or basal level of cAMP before stimulation. Thus, under the experimental condition with Hela cells, pre-exposure of cells to ethanol does not alter the machinery involved in the acute effect of ethanol on cAMP signaling. It has been shown that chronic exposure to ethanol reduces cAMP signaling in

many experimental systems (Tabakoff and Hoffman, 1998). In fetal rat hypothalamic neurons, ethanol exposure reduces cAMP content and mRNA levels of ACs (Chen et al., 2006). One of the reasons why we did not observe changes in cells pre-exposed to ethanol could be due to the fact that transfected GPCR and AC were employed in the experiments instead of endogenously expressed counterparts.

Perhaps most interesting, is our observation of a complete reversal of the alcohol effect when cells are exposed to ethanol for only 5 seconds as opposed to 2 minutes. There appears to be an inhibitory effect of ethanol upon very short exposure (5 seconds), which is distinctly different from the ethanol response we have observed in 2-minute ethanol exposure. Past studies utilizing biochemical analysis did not have the temporal resolution required to see such an effect. Although this newly discovered inhibition may not have any physiological relevance as opposed to the enhancement of AC activity, it will help elucidate the mechanism by which ethanol affects the function of AC and other proteins. This inhibitory effect is AC isoform specific. In addition, the effect seems to be independent of GPCR employed, suggesting that the site of ethanol action is the AC7 protein. The cAMP peak was reached ~20 seconds after the start of drug application. Judging from the speed of perfusion (~10 ml/min) and the volume in the perfusion chamber (<200 μ l), all drugs, including ethanol should be cleared from the chamber within a few seconds. However, AC7 activity remained lower even after ethanol clearance, which was very clear without cAMP degradation in the presence of IBMX (Fig. 6B). The observed inhibition can be achieved by two different mechanisms: 1) by inhibiting the activity of each AC7 molecule, 2) by reducing the number of active AC7 molecules. Our current results are not enough to distinguish which mechanism is in effect. On the other hand, the enhancing effect of ethanol observed during longer exposure is due to an increase in each AC7 protein's activity. This was demonstrated using recombinant AC7 protein expressed in bacteria (Dokphrom et al., 2011). Ethanol was shown to increase $V_{\max}$ value of AC7. Overall, our observations in this report and previous studies suggest that ethanol increases Gs-stimulated activity of AC7 by interacting with the AC7 protein directly. This enhancement of activity should be accompanied by a change in the conformation of the protein. It appears that the conformational change goes through a short transient state where AC7 is less sensitive to activation by Gs.

In summary, real-time measurement of cAMP by FRET based cAMP sensor confirmed previous studies that demonstrated AC isoform specific and concentration dependent enhancing effects of ethanol and provided detailed time courses of cAMP changes in different subcellular compartments. Furthermore, with this new method we discovered a transient, AC isoform specific inhibitory effect of ethanol, which was not observed with conventional methods due to poor spatiotemporal resolution. The real-time monitoring of cAMP provides invaluable tools and complements conventional methods to elucidate the mechanism by which ethanol alters cAMP signaling.

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Fig. 1. Subcellular compartment specific expression of Epac1-camps. Epac1-camps directed to a specific subcellular compartment (A; cytoplasm, B; nucleus, C; plasma membrane) was expressed in HeLa cells and fluorescent images (YFP) were captured as described in MATERIALS AND METHODS. Scale bar; 10 μ m.

Fig. 2.

The effect of ethanol on DA stimulated cAMP in cells expressing AC7 and DRD1A. A) FRET based real-time monitoring of cAMP in the cytoplasm. Normalized nFRET values are plotted over time. The addition of 0.2 μ M DA and ethanol was indicated with a bar on the top of the graph. Data are presented as mean $\pm$ SEM (DA; n=19, DA + EtOH 100 mM;

n=21, DA + EtOH 200 mM; n= 16). In the presence of ethanol (100 or 200 mM), the lowest value of nFRET is significantly smaller than that of DA alone ($p<0.001$, Student's *t*-test). Also the values at plateau (values at last 10 time points) are significantly smaller in the presence of ethanol ($p<0.001$, two-way repeated-measures ANOVA). B) cAMP accumulation assay with cells expressing AC7. The cells were stimulated with 10 μ M DA together with the indicated concentration of ethanol for 30 seconds. The percentage increases of cAMP accumulation over that stimulated with DA alone are plotted. Data are presented as mean $\pm$ SEM (n=4). * Value is significantly higher than DA alone ($p<0.001$, One-way ANOVA).

Fig. 3.

The effect of ethanol on DA stimulated cAMP in cells expressing AC3. Real-time change in cAMP in the cytoplasm of the cells expressing AC3 and DRD1A is monitored by nFRET plotted over time. The bar on the top of the graph indicates the duration of DA (0.2 μ M) and ethanol treatment. Data are presented as mean $\pm$ SEM (DA; n=17, DA + EtOH 100 mM; n=19, DA + EtOH 200 mM; n= 13). There are no significant differences in nFRET values at lowest point or at the plateau in the presence of ethanol compared with those in the absence of ethanol.

Fig. 4.

The effects of ethanol on cAMP at the plasma membrane and in the nucleus. Cells were stimulated with $0.2 \mu\text{M}$ DA $\pm$ 200 mM ethanol for 2 minutes as indicated by a bar at the top of the graphs. A) FRET recording of Epac1-camps targeted to the plasma membrane in cells

expressing AC7 and DRD1A; mean $\pm$ SEM (DA; n=17, DA+EtOH; n=13). In the presence of ethanol, the lowest value of nFRET is significantly smaller than that of DA alone, ($p < 0.05$, Student's *t*-test). B) FRET recording of Epac1-camps targeted to the nucleus in cells expressing AC7 and DRD1A; mean $\pm$ SEM (n=12). In the presence of ethanol; mean $\pm$ SEM (n=13), the lowest value of nFRET is significantly smaller than that of DA alone ($p < 0.01$, Student's *t*-test). Also the values at plateau (values at last 10 time points) are significantly smaller in the presence of ethanol ($p < 0.05$, two-way repeated-measures ANOVA). C) FRET recording of Epac1-camps targeted to the plasma membrane in cells expressing AC3 and DRD1A; mean $\pm$ SEM (DA; n=24, DA+EtOH; n=7). D) FRET recording of Epac1-camps targeted to the nucleus in cells expressing AC3 and DRD1A; mean $\pm$ SEM (DA; n=13, DA+EtOH; n=13).

Fig. 5.

The effect of 24-hour exposure to ethanol on cAMP in cells expressing AC7 and DRD1A. FRET recording of Epac1-camps targeted to the cytoplasm in cells expressing AC7; mean $\pm$ SEM (DA; $n=19$, DA+EtOH; $n=15$, Exp-24h-DA; $n=9$, Exp-24h-DA+EtOH; $n=14$). Cells were incubated with either regular medium or regular medium plus 50 mM ethanol for 24 hours (Exp-24h) before FRET recording. During the recording cells were treated with 0.2 μ M DA $\pm$ 200 mM ethanol for 2 minutes as indicated by a bar at the top of the graphs. When cells were not treated with ethanol prior to the recording ethanol caused a significant decrease in nFRET values at minimum ($p<0.001$, Student's t -test) and at plateau ($p<0.001$, two-way repeated-measures ANOVA). Similarly when cells were treated with ethanol for 24 hours prior to the recording addition of ethanol with DA decreased nFRET values compared with DA alone (minimum; $p<0.05$, plateau; $p<0.001$). However, 24-hour treatment with ethanol did not change nFRET values at minimum and at plateau significantly (DA vs. Exp-24h-DA and DA+EtOH vs. Exp-24h-DA+EtOH).

Fig. 6.

Effect of short stimulation with DA and ethanol on cAMP in cells expressing AC7. A) FRET recording of Epac1-camps targeted to the cytoplasm in cells expressing AC7 and DRD1A; mean $\pm$ SEM (DA; n=21, DA+EtOH; n=13). A bar on top of the graph indicates 5-second addition of 0.2 μ M DA $\pm$ 200 mM ethanol. Ethanol significantly increased the nFRET value at minimum compared to that of DA alone ($p = <0.001$, Student's t -test). B) FRET recording in the presence of 500 μ M IBMX; mean $\pm$ SEM (DA; n=23, DA+EtOH; n=16). The treatment of the cells was similar to that shown in A) except IBMX was added at the same time as DA or DA+EtOH and stayed during the recording as indicated with grey bar on top of the graph. The decrease of nFRET value was significantly attenuated in the presence of ethanol $p = <0.001$, Student's t -test).

C **D**

Fig. 7.
Effect of ethanol on cAMP stimulated by different GPCRs. FRET recording of Epac1-camps targeted to the cytoplasm in cells expressing AC7 and one exogenous GPCR stimulated with

the respective agonist and 200 mM ethanol (A–F). Duration of stimulation is indicated with a bar on top of the graphs. A) ADORA2A, 10 μ M CGS21680 (Ligand; n=21, Ligand+EtOH; n=16). B) ADRB2, 10 μ M isoproterenol (Ligand; n=15, Ligand+EtOH; n=19). C) HRH2, 10 μ M amthamine dihydrobromide (Ligand; n=10, Ligand+EtOH; n=17). D) ADORA2A, 10 μ M CGS21680 (Ligand; n=15, Ligand+EtOH; n=12). E) ADRB2, 10 μ M isoproterenol (Ligand; n=14, Ligand+EtOH; n=18). F) HRH2, 10 μ M amthamine dihydrobromide (Ligand; n=15, Ligand+EtOH; n=28). A), B), C) Ethanol significantly decreased nFRET values (minimum: AB; $p < 0.05$, C; $p < 0.01$, plateau: A; $p < 0.05$, B–C; $p < 0.001$). D) Ethanol significantly attenuated changes in nFRET values (minimum: $p < 0.05$, plateau: A; $p < 0.01$). E–F) Ethanol had no significant effects or remain unchanged when stimulated with respective agonists for 5 seconds (minimum; E: $p = 0.167$, F: $p = 0.146$, plateau; E: $p = 0.189$, F: $p = 0.34$).

Fig. 8.

Effects of ethanol on 5-second DA stimulation on cAMP. Cells were treated either with 0.2 μ M DA alone or 0.2 μ M DA plus 200 mM ethanol for 5 seconds while constantly perfused with KRH buffer (KRH-DA, KRH-DA+EtOH). The third plot was from cells treated with 0.2 μ M DA+EtOH for 5 seconds while perfused with KRH buffer containing 200 mM ethanol starting 2 minutes before DA+EtOH application (KRH: EtOH-DA+EtOH). A bar indicates addition of DA $\pm$ EtOH. A) FRET recording of Epac1-camps targeted to the cytoplasm in cells expressing AC7 and DRD1A; mean $\pm$ SEM (KRH-DA; n=16, KRH-DA+EtOH; n=14, KRH: EtOH-DA+EtOH; n=17). Ethanol significantly increased the nFRET

value at minimum compared to that of DA alone ($p < 0.01$) when added together with DA, while ethanol decreases the nFRET value ($p < 0.01$) when added 2 minutes before DA addition. There were no differences in nFRET values at plateau (last 10 time points) regardless of the treatment. The nFRET values at plateau and before addition of DA were similar. B) FRET recording of Epac1-camps targeted to the cytoplasm in cells expressing AC3 and DRD1A; mean $\pm$ SEM (KRH-DA; $n=7$, KRH-DA+EtOH; $n=6$, KRH: EtOH-DA+EtOH; $n=6$). Ethanol did not have any significant effect. The nFRET values at plateau and before addition of DA were similar.