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# Phosphorylation Cascades Control the Actions of Ethanol on Cell cAMP Signalling

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Alcohol · Adenylyl cyclase · cAMP · Protein kinase A · Protein kinase C

**Abstract**
Our studies indicate that, in the presence of particular isoforms of adenylyl cyclase (i.e., type 7 AC), moderately intoxicating concentrations of ethanol will significantly potentiate transmitter-mediated activation of the cAMP signaling cascade. Activation of this signaling cascade may have important implications for the mechanisms by which ethanol produces intoxication, and/or for the mechanisms of neuroadaptation leading to tolerance to, and physical dependence on, ethanol. We initiated a series of studies to investigate the phosphorylation of AC7 by PKC, the role of this phosphorylation in modulating the sensitivity of AC7 to activation by Gsα, and the PKC isotype(s) involved in the phosphorylation of AC7. The T7 epitope-tagged AC7 expressed in Sf9 and HEK293 cells was found to be phosphorylated in vitro by the catalytic subunit of PKC. Treatment of AC7-transfected HEK293 cells with phorbol dibutyrate (PDBu) or ethanol increased the phosphorylation of AC7 and its responsiveness to Gsα. In human erythroleukemia (HEL) cells, which endogeneously express AC7, ethanol and PDBu increased AC activity stimulated by $PGE_1$. The potentiation by both PDBu and ethanol was found to be sensitive to the PKC δ-selective inhibitor, rottlerin. The potentiation of AC activity by ethanol in HEL cells was also selectively attenuated by the RACK inhibitory peptide specific for PKC δ, and by expression of the dominant negative, catalytically inactive, form of PKC δ. These data demonstrate that AC7 can be phosphorylated by PKC, leading to an increase in functional activity, and ethanol can potentiate AC7 activity through a PKC δ-mediated phosphorylation of AC7.



The G-protein-mediated signal transduction pathways that use adenylyl cyclase (AC) as an effector are among the most common signal transduction pathways in both neuronal and non-neuronal cells. Neurotransmitters bind to Gs-coupled receptors and promote the dissociation of the heterotrimeric Gs protein and the exchange of GTP for GDP bound to the Gsα subunit. The Gsα subunit with GTP bound to it then activates the AC. The activated AC generates the second messenger cyclic adenosine 3′5′-monophosphate (cAMP), which in turn activates the cAMP-dependent protein kinase A (PKA). PKA is known to phosphorylate and regulate several target proteins in

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different intracellular regions from the plasma membrane to the nucleus. Several studies have shown that ethanol *acutely* potentiates Gs-stimulated AC activity in various whole cell and membrane preparations [1, 50, 53], and it is not surprising that consequences of the stimulatory effects of ethanol on the Gs-AC signal transduction pathway can be observed in the plasma membrane as well as in the nucleus. In studies by Palmer's group, the cAMP-generating system has been shown to be a controlling element in the modulation of ethanol sensitivity of $GABA_A$ receptor-coupled ion channels [10, 26]. Activation of AC through β-adrenergic receptors potentiated Purkinje neuron responses to GABA, and sensitized GABA responses to the potentiating effect of ethanol [10, 26]. Since ethanol can increase β-adrenergic receptor-stimulated AC activity, this pathway suggests a mechanism by which ethanol can produce a 'a feedforward' sensitization of $GABA_A$ receptors on cerebellar Purkinje neurons to the action of ethanol. Acute exposure of rats to ethanol has also been demonstrated to activate AC signal transduction from the membrane to the nucleus. Increased cAMP production at the plasma membrane increased the phosphorylation of CREB in the nucleus of striatal and cerebellar tissues [60, 61]. In addition, Moore et al. [39] demonstrated in a recent behavioral study using *Drosophila* mutants that cAMP levels, and consequently PKA activity, determined the sensitivity of the flies to the intoxicating effect of ethanol.

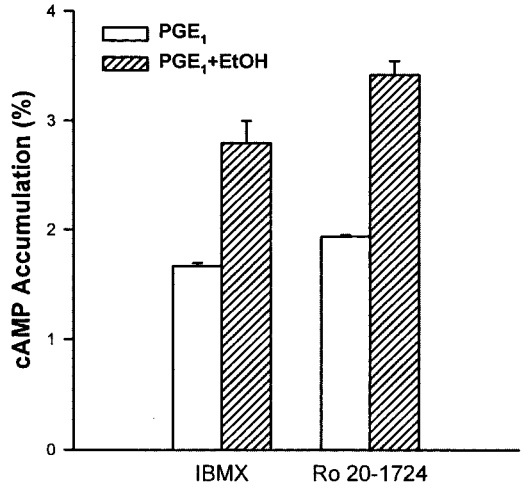
Adaptation of the AC system occurs after *chronic* exposure to ethanol, resulting in reduced responsiveness of AC to different stimuli, and leading to lowered cAMP production and PKA activity. These mechanisms have been suggested to play a role in behavioral tolerance to ethanol [9, 53].

Observations that the actions of ethanol on AC activity are evident primarily under conditions where Gs (the stimulatory G protein) is also activated have led to the hypothesis that ethanol acts to promote the rate of activation of Gs protein and/or to sensitize AC to the action of the Gs protein [19]. On the other hand, ethanol has also been shown to inhibit nucleoside transporters which internalize extracellular adenosine [8], and it has been proposed that the ethanol-induced increases in levels of extracellular adenosine lead to activation of AC through the interaction of adenosine with Gs-coupled $A_2$ adenosine receptors [8].

One of the difficulties in studying the mechanism of action of ethanol on AC activity in native tissues is the fact that ACs are a gene family. Currently, nine different genes that code for ACs (AC1–AC9) have been identified, and some of these genes also produce multiple splice variants [24, 51, 52]. Most cell types, including neurons, probably express two or more AC isoforms [16, 45]. In fact, practically all AC isoforms are expressed in brain, and each isoform has a unique but overlapping expression pattern, as demonstrated by in situ hybridization studies [38]. Since there is significant functional diversity in the family of ACs [24, 51, 52], it is important to realize that the complement of the ACs in a given cell type, as well as the ACs' subcellular localization and association with regulators and modulators, will probably determine how ethanol will influence AC signal transduction pathways both acutely and chronically.

All currently known AC isoforms can be activated through Gs-coupled receptors, although the conditions, magnitude and mechanisms of such activation vary from isoform to isoform [15, 24, 51, 52]. Similarly, ethanol's ability to potentiate Gs-stimulated AC activity varies among isoforms, with AC7 being the most sensitive to ethanol of all tested isoforms [64]. For instance, 50 m*M* ethanol produced a 56% increase in $PGE_1$-stimulated cAMP production in HEK293 cells transfected with type 7 AC [65], while activation of cAMP production in HEK293 cells transfected with other AC family members was, at best, one half to one third of this value. We wished to determine the characteristics of AC7 that result in the enhanced sensitivity to the potentiating effect of ethanol. Since in our prior work and the work of others, 3-isobutyl-1-methylxanthine (IBMX) has been used routinely as an inhibitor of phosphodiesterase to protect newly formed cAMP, and since IBMX can also act as a competitive antagonist at $A_2$ adenosine receptors, it became initially important for us to examine whether the actions of IBMX at the adenosine receptors were contributing somehow to our witnessed results regarding ethanol potentiation of AC7 activity.

To initiate the examination of the role of adenosine and adenosine $A_2$ receptors in the actions of ethanol on AC, we examined the effect of ethanol on AC activity in HEK cells transfected with AC7 in the presence of Ro 20-1724, a phosphodiesterase inhibitor lacking activity at the adenosine $A_2$ receptor. Figure 1 demonstrates that ethanol had equivalent effects in the presence of Ro 20-1724 or IBMX. To further examine the possible role of adenosine in the actions of ethanol, we examined the ability of adenosine per se to increase the accumulation of cAMP in HEK293 cells transfected with AC7. Adenosine (10 μ*M*) produced a small increase in cAMP accumulation in the AC7-transfected HEK293 cells (0.048%), whereas $PGE_1$ (10 μ*M*), which we routinely use for our studies [65], pro-

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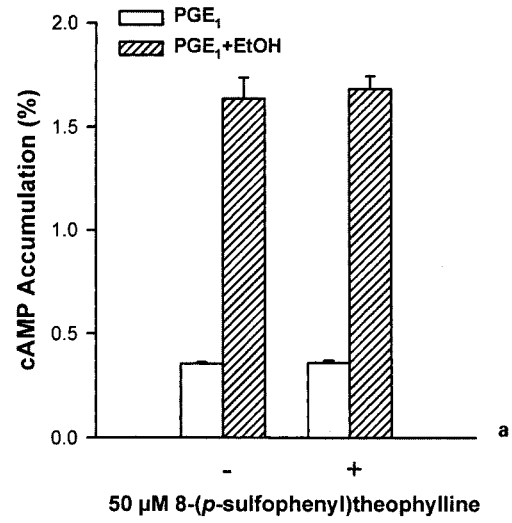
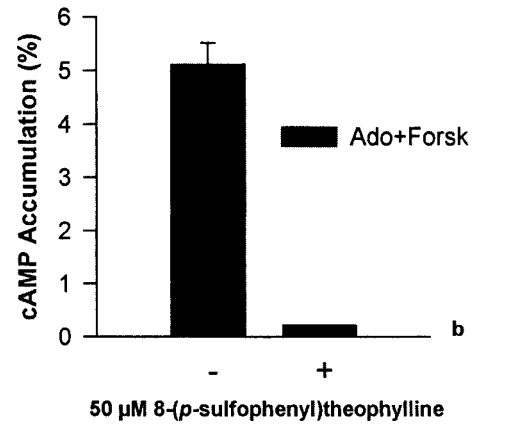



**Fig. 1.** Effects of IBMX and Ro 20-1724 on ethanol-induced increases in cAMP production. cAMP accumulation was measured in HEK293 cells transfected with AC7. Cells were preincubated, prior to $PGE_1$ addition, for 10 min with either 500 μ*M* IBMX or 500 μM Ro 20-1724, and incubated for 5 min with 10 μ*M* $PGE_1$ in the presence or absence of 200 m*M* ethanol, as indicated. The effects of $PGE_1$ and $PGE_1$ + ethanol are reported as cAMP accumulation.

**Fig. 2.** Effect of an adenosine receptor agonist on cAMP accumulation. **a** HEK293 cells transfected with AC7 were incubated for 5 min with 10 μ*M* $PGE_1$ ± 200 m*M* ethanol in the presence or absence of 50 μ*M* 8-(*p*-sulfophenyl) theophylline, as indicated. cAMP accumulation was measured. The stimulation by ethanol was 358.8 ± 28.3% in the absence and 366.8 ± 17.2% in the presence of this adenosine antagonist. **b** The effect of the adenosine receptor antagonist on the actions of adenosine, per se, was examined by incubating the cells with 10 μ*M* adenosine (Ado) plus 10 μ*M* forskolin (Forsk) for 5 min.

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duced a much higher increase (0.35%). In order to accentuate the effect of adenosine acting as an agonist at $A_2$ adenosine receptors, adenosine was added together with forskolin (fig. 2). A synergistic activation of AC activity was demonstrated when the Gs-coupled $A_2$ receptor agonist (adenosine) was added with forskolin to cells expressing AC7. The adenosine receptor antagonist [8-(*p*-sulfophenyl)theophylline] blocked the effect of adenosine on forskolin-activated AC activity (fig. 2b), but had no effect on $PGE_1$-stimulated AC activity or the potentiation of this activity by ethanol (fig. 2a). Because of its poor membrane permeability, 8-(*p*-sulfophenyl)theophylline would not be expected to inhibit intracellular events under our assay conditions.

As already mentioned, prior work [8] had suggested that ethanol acts to enhance extracellular adenosine levels, and it has been proposed that increases in AC activity in the presence of ethanol are wholly generated through the increased activation of $A_2$ adenosine receptors by the accumulated adenosine. Our current studies illustrate little or no role for adenosine in the actions of ethanol on $PGE_1$-mediated increases in cellular cAMP. A possible interpretation of the prior findings on the importance of endogenous adenosine in ethanol-induced actions on

cAMP signaling [8], is that activation of a Gs-coupled receptor is critical for evidencing ethanol-induced actions on AC activity. If an appropriate exogenous transmitter substance (e.g. $PGE_1$) is not added to a cellular assay system, ethanol would have no effect on cAMP levels unless an endogenous transmitter (such as adenosine) accumulated to levels sufficient to stimulate the cognate (adenosine $A_2$) Gs-coupled receptor.

We have additionally argued that the actions of ethanol on cAMP generation transcend the actions of ethanol on the Gs protein activation process and involve an ethanol effect on the Gs-AC interaction. Our contention was based on the already-stated fact [64] that certain members of the AC enzyme family were substantially responsive to the actions of ethanol while other members were not responsive. Since all members of the AC family of enzymes can, under specific conditions, be activated by Gsα [52, 54], the characteristics of a particular AC must contribute to the degree of ethanol potentiation of the catalytic activity of AC, irrespective of the actions of ethanol on the Gs protein.

An interesting feature of AC7 is that it is one of the AC isoforms that are sensitive to the effects of phorbol esters, through activation of protein kinase C (PKC) [17, 22, 59, 62]. Our studies showed that both the effects of ethanol and phorbol esters on AC7 are blocked by bisindolylmaleimide and staurosporine, a relatively selective PKC inhibitor and a Ser/Thr kinase inhibitor, respectively. These data suggested a role for PKC in the actions of ethanol on AC7. Further work in our laboratory [46] demonstrated that prolonged preincubation of cells (20 h) with a phorbol ester blocked the actions of ethanol on AC in human erythroleukemia (HEL) cells, which express AC7 as their primary endogenous AC isoform [16]. Although little published evidence exists for direct phosphorylation of AC7 by PKC, direct phosphorylation of the closely related AC2 by PKC α has been demonstrated [67]. Since both AC7 and AC2 have PKC consensus sites in functionally relevant regions, i.e. AC catalytic domains ($C_{1a}$ and $C_{2a}$) and putative regulatory regions ($C_{1b}$) [24, 54, 56, 66], we hypothesized that AC7 activity can be regulated by PKC through direct phosphorylation, and that certain of these phosphorylation events may be involved in the actions of ethanol on AC7.

To determine whether or not AC7 could itself be a target (substrate) for PKC phosphorylation, a His- and T7-epitope tagged AC7 expression vector was constructed which would allow for efficient purification by immunoprecipitation. The tagged AC7 was overexpressed in Sf9 insect cells by infection with a recombinant baculovirus. The membranes from AC7- and control-infected Sf9 cells were solubilized, and the AC7 protein was immunoprecipitated using the monoclonal anti-T7 antibody. Western blot and autoradiographic evidence for in vitro phosphorylation of AC7 protein by PKC was obtained after incubation of the immunoprecipitated AC7 with rat brain PKC catalytic subunits in the presence of [$\gamma$-$^{32}$P]ATP. A phosphorylated band with the same mobility characteristics as AC7 was noted after polyacrylamide gel electrophoresis, while no phosphorylated band was noted in control assays which contained no AC7. Given these results, we also investigated whether ethanol could promote the phosphorylation of AC7 by PKC. AC7-transfected HEK293 cells were simultaneously incubated with $PGE_1$ and ethanol for 2–5 min, and the cell membranes were prepared under conditions which would preserve the phosphorylation state of AC7. We then performed a 'back' phosphorylation experiment by monitoring the extent of AC7 phosphorylation by PKC in vitro using the membranes of the ethanol-treated and control cells. The reduced phosphorylation of AC7 by PKC in vitro ('back' phosphorylation) indicated that ethanol had promoted phosphorylation of AC7 at PKC 'substrate' sites.

To show functional significance for the enhanced phosphorylation of AC7 seen with ethanol, AC7 activity was assayed in membranes that were prepared from ethanol-, PDBu-, or control vehicle-treated AC7-transfected HEK293 cells. When constitutively active Q227L-Gsα was added to the membranes from AC7-transfected HEK293 cells that had been pretreated with PDBu or vehicle immediately prior to membrane preparation, the activity of expressed AC7 in the presence of Q277-Gsα was significantly higher in membranes of PDBu-treated cell membranes than in vehicle-treated cell membranes. This indicated that the phosphorylation of AC7 by PKC may change the responsiveness of the enzyme to Gsα. As for PDBu, ethanol exposure of AC7-transfected cells also increased the responsiveness of the enzyme to added Q227L-Gsα, when tested in the cell-membrane-containing assay for AC activity.

There is a significant amount of diversity in the family of PKCs, which consists of at least 11 different isoforms [41]. The PKC family can be divided into three subfamilies: the conventional PKCs (cPKC: α, βI, βII and γ) are $Ca^{2+}$ dependent and activated by phosphatidylserine and diacylglycerol (DAG) and its analogs, the phorbol esters; the novel PKCs (nPKC: δ, ε, η, θ and μ) are $Ca^{2+}$ *in*dependent, but activated by phosphatidylserine and DAG and phorbol esters; the atypical PKCs (aPKC: λ and ζ) are not activated by $Ca^{2+}$ or by DAG or phorbol esters [41]. An

important question is how the specificity of regulation (phosphorylation) of a target protein by a kinase arises. Signal transduction pathways, such as the receptor-G protein-AC pathway, appear to be organized into multi-element complexes by scaffold and anchoring proteins that ensure functional integrity, efficiency and spatio-temporal control of signaling [3, 57]. The specificity of PKC actions is thought to originate from the presence of PKC-isoform-specific anchoring proteins [36, 37] as well as the presence of PKC-isoform-specific substrate sequences [40] in the target proteins, such as AC7. It is probable that *both* the PKC-isoform-specific anchoring protein and the nature of the substrate sequence together determine the efficiency and level of phosphorylation of a protein and the functional consequences of the phosphorylation of a given protein such as an AC. Having shown that AC7 can be directly phosphorylated by PKC, and that ethanol can promote this phosphorylation, we next investigated the isoforms of PKC responsible for the effect of ethanol. For these experiments, we used HEL cells.

HEL cells express mRNA for AC6, AC7, and the ethanol-insensitive AC3 [64]. AC7 mRNA is by far the most abundant species of AC mRNA expressed in HEL cells [16]. Furthermore, using an AC2 family antibody, an immunoreactive band in the range of 110 kD was identified in the membrane fraction of HEL cells, which corresponded in size to an immunoreactive band of AC7-infected Sf9 cells and AC7-transfected HEK293 cells. Therefore, HEL cells seemed appropriate for further investigation of the role of PKC in ethanol potentiation of AC activity. Gö-6976 has been shown to selectively inhibit the conventional PKCs and PKC μ, while rottlerin selectively inhibits PKC δ and θ [13, 32, 58]. Pretreating HEL cells with increasing concentrations of Gö-6976 had little or no effect on the potentiation of $PGE_1$-stimulated AC activity by either PDBu or ethanol. However, when HEL cells were pretreated with rottlerin, the potentiation of AC activity by both ethanol and PDBu was inhibited in a concentration-dependent fashion. For example, with 2.5 μ*M* rottlerin in the assay, the potentiation by ethanol was reduced from 83 ± 10 to 28 ± 7%, and the potentiation by PDBu was reduced from 112 ± 11 to 59 ± 12%.

The role of a PKC anchoring protein is to target the appropriate PKC isoform in the vicinity of its substrate [36, 37]. So far only two PKC anchoring proteins, or receptors for activated C kinase (RACKs), have been cloned. RACK1 is selective for PKC β [36, 48, 49], and the second RACK is selective for PKC ε [7]. Interestingly, the PKC anchoring RACK proteins are structurally related to G protein β subunits [48] that stimulate the activity of AC2-family enzymes, including AC7 [55, 63]. Peptides containing specific sequences within the N-terminal V-1 regions which are unique to the various PKCs have been recently used to competitively inhibit the binding of PKCs to the RACK binding proteins. These peptides have been shown to be PKC-isotype-specific, and to have little cross-reactivity. When HEL cells were pretreated with RACK inhibitory peptides (kindly provided by Dr. Daria Mochly-Rosen, Stanford University, Stanford, Calif., USA) prior to stimulation by $PGE_1$, the potentiation of AC activity by ethanol was attenuated by about 50% (from 68 ± 16 to 36 ± 3%, $p < 0.05$, Student's t test) by the RACK inhibitory peptide specific for PKC δ (δV1-1). In contrast, the inhibitory peptide for PKC ε (εV1-2) or a control (scrambled sequence) peptide had no effect (ethanol potentiation was 62 ± 11%). The potentiation by PDBu was also significantly reduced by the δV1-1 peptide, whereas the PKC ε peptide and scrambled control peptide were without effect.

Overexpression of dominant negative (DN), catalytically inactive, forms of PKC has previously been shown to competitively inhibit the activity of the targeted PKC isoform, either by displacing the endogenous PKC from its substrate, and/or by competing with the endogenous PKC for co-factors and substrates. The DN construct for PKC δ has a lysine in position 376 mutated to a methionine within the ATP binding site, making it catalytically inactive [25b]. Both the wild-type and DN constructs of PKC δ have been incorporated into separate replication-deficient adenovirus (Ad5 DL312) vectors, kindly provided to us by Dr. Trevor Biden (Garvan Institute of Medical Research, Sydney, Australia). HEL cells were infected with the viral vectors carrying either the wild-type or DN form of PKC δ at two different adenovirus titers, $10 \times 10^3$ or $50 \times 10^3$ particles/cell. HEL cells infected with the DN-PKC δ adenovirus demonstrated a 174% increase in immunoreactive PKC δ protein, representing the expression of the DN mutant PKC δ in addition to the endogeneous expression of wild-type PKC δ. When HEL cells were assayed for AC activity 20 h after viral infection, the potentiation of $PGE_1$-stimulated AC activity by 100 m*M* ethanol was no longer statistically significant in cells infected with the DN-PKC δ adenovirus, at either virus titer. At the higher virus titre, the potentiation of ethanol was reduced to 16 ± 7%, as compared to 52 ± 18% in cells infected with the wild-type PKC δ adenovirus.

Our previous results [46] ruled out the involvement of the atypical PKCs in the effect of ethanol by demonstrating that the downregulation of the classical and novel PKCs by prolonged exposure to phorbol esters eliminated

ethanol potentiation of $PGE_1$-stimulated AC activity in HEL cells. Our current results also demonstrated that the PKC inhibitor, Gö-6976, which is selective for the classical PKCs, did not reduce the potentiation of AC activity produced by ethanol or PDBu. This lack of effect was evident at Gö-6976 concentrations that were 50- to 500-fold higher than the published Ki values for PKC α, β and μ [14, 32]. The sum of these data indicated the lack of involvement of the atypical and classical PKCs, as well as PKC μ, in the effects of ethanol or PDBu on AC activity.

On the other hand, our pharmacological and molecular biological studies suggested a role for a novel PKC (PKC δ) in ethanol and phorbol ester potentiation of agonist-stimulated AC7 activity. First, rottlerin has been shown to selectively inhibit PKC δ and θ function [58], and to selectively inhibit the PKC δ and θ isozymes (Ki = 3–6 μ*M*) with 10-fold greater potency than the α, β and γ isoforms and a 20- to 30-fold greater potency than the ε, η and ζ isoforms [2, 14]. Rottlerin has been used to implicate PKC δ in many cellular functions, such as the regulation of cell growth and differentiation, apoptosis, and tumor development [6, 29, 44, 47]. Our results demonstrated a reduced potentiation of AC activity by either PDBu or ethanol even in the presence of a concentration of rottlerin below its Ki for inhibition of PKC δ [13]. However, rottlerin inhibition alone could not distinguish between the involvement of PKC δ and θ, both of which are expressed in HEL cells. Rottlerin has also been shown to affect CaM kinase III and Trk receptor activity [42]. Thus, additional experiments were performed using a novel class of selective peptide PKC inhibitors.

A RACK inhibitory protein similar to the one we used was previously shown to prevent translocation of PKC δ upon cell stimulation [23]. A related peptide has also been used to demonstrate the involvement of PKC δ in mediating ethanol-induced upregulation of L-type $Ca^{2+}$ channels [11]. Our results, demonstrating that the PKC δ RACK inhibitory peptide, but not the PKC ε peptide or a scrambled peptide, inhibit PDBu and ethanol-induced potentiation of AC activity, further support a role for PKC δ in the action of ethanol.

Finally, catalytically inactive DN forms of various PKC isotypes have also been successfully used as specific inhibitors of PKC actions, and a recent study used a DN PKC to delineate opposing effects by two different PKC isotypes, δ and α [20]. We can conclude from our studies using DN PKC δ that PKC δ is in large part responsible for the potentiation by ethanol of prostanoid receptor/Gs protein-coupled AC activity in HEL cells. The fact that some potentiation remained even in the presence of the RACK inhibitor peptide and the dominant-negative PKC δ isoform may, however, argue for an additional mechanism for the effects of ethanol on AC activity.

Only two prior studies have implicated the novel PKCs in regulating AC activity. Inhibition of AC6 activity by a novel PKC during adenosine A2a receptor desensitization in PC12 cells was implied by the lack of $Ca^{2+}$ dependence of this process and the effects of overexpression of PKC δ or ε in the PC12 cells [25a]. The potentiation of $PGE_1$-stimulated AC2 activity by phorbol-12 myristate, 13 acetate in macrophages was also suggested to be due to the novel family of PKCs [28]. On the other hand, atypical PKCs were reported to mediate lysophosophatidic acid potentiation of $PGE_1$-stimulated AC2 activity in these same cells [27]. Neither study by Lin et al. [27, 28] showed evidence of direct phosphorylation of AC, and it is not clear which AC isoforms besides AC2 are present in macrophages. Given our work showing that AC7 can be directly phosphorylated by PKC, it is of interest that in addition to the putative PKC phosphorylation consensus sites in the catalytically relevant cytoplasmic loops of AC7, putative sites for which the novel PKCs show preference [40] are present within the Gsα binding domains. This is particularly intriguing since we have shown that phosphorylation of AC7 by PKC resulted in greater sensitivity of the enzyme to stimulation by Gsα. In early examinations of the actions of ethanol on AC activity, we demonstrated that ethanol increases the rate of activation of Gs [30]. If an increased availability of activated Gsα* occurs in the presence of phosphorylated AC7, our results would predict a significant activation of this AC isoform, which could explain why AC7 is the most sensitive to the actions of ethanol.

In addition to our demonstration of PKC δ involvement in ethanol modulation of AC activity, the novel family of PKCs has been implicated in various other acute and chronic effects of ethanol. Short-term ethanol exposure of NG108-15 cells can alter the subcellular localization and thus presumably the function of PKC δ and ε [12]. PKC ε has been found to be involved in ethanol-induced cardioprotection [5, 35], and the acute behavioral effects of ethanol mediated through $GABA_A$ receptors are dampened in PKC ε knockout mice [18]. Chronic ethanol exposure upregulated the density and function of L-type $Ca^{2+}$ channels via a PKC-δ-dependent mechanism [4], while the upregulation of N-type $Ca^{2+}$ channels by chronic ethanol treatment was recently found to be attributable to PKC ε [33]. Chronic ethanol treatment of PC12 cells also enhanced nerve growth factor-induced neurite outgrowth and activation of MAP kinases by a PKC ε-dependent mechanism [21].

The exact mechanism or mechanisms by which ethanol activates PKC δ to alter AC7 activity remains unknown. Although previous data have indicated that ethanol has little if any direct stimulatory effect on the catalytic activity of PKC in vitro [31, 34], ethanol could modulate the interaction between, and the co-localization of, AC7 and PKC δ. A pool of endogenously active DAG-sensitive PKCs residing at the cell membrane has been recently described in a number of cell types, including HEL cells [4]. Other PKCs were localized primarily in the cytosol. In murine erythroleukemia cells, PKC δ was found to be predominantly membrane associated, and in a constitutively active state [43]. Thus, the acute effects of ethanol on AC activity, which can occur in less than 1 min [46], could be mediated through a pool of already activated, membrane-bound PKC δ. Ethanol could promote a conformational change in AC which provides or enhances availability of a site(s) for PKC-mediated phosphorylation, or ethanol could promote the association of AC with PKC δ within a transducisome complex [57].

The conclusions from our experiments, overall, are that under basal conditions, PKC δ maintains AC7 in a modestly phosphorylated form to maintain its responsiveness to Gsα. In the presence of ethanol, the phosphorylation of AC7 by PKC δ is enhanced. The more phosphorylated form of this AC isoform becomes more sensitive to activated Gsα and, in this way, ethanol enhances receptor-mediated signaling through the AC system. The increased levels of cAMP during such a signaling process will produce a greater effect on PKA, and greater modification of downstream effectors dependent on cAMP signaling.

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## References

1 Bode DC, Molinoff PB. Effects of ethanol in vivo on the beta adrenergic receptor-coupled adenylate cyclase system. J Pharmacol Exp Ther 246:1040–1047;1988.
2 Braiman L, Alt A, Kuroki T, Ohba M, Bak A, Tannenbaum T, Sampson SR. Protein kinase C delta mediates insulin-induced glucose transport in primary cultures of rat skeletal muscle. Mol Endocrinol 13:2002–2012;1999.
3 Bray D. Protein molecules as computational elements in living cells. Nature 376:307–312; 1995.
4 Chakravarthy BR, Whitfield JF, Durkin JP. Inactive membrane protein kinase Cs: A possible target for receptor signalling. Biochem J 304:809–816;1994.
5 Chen CH, Gray MO, Mochly-Rosen D. Cardioprotection from ischemia by a brief exposure to physiological levels of ethanol: Role of epsilon protein kinase C. Proc Natl Acad Sci USA 96:12784–12789;1999.
6 Corbit KC, Foster DA, Rosner MR. Protein kinase C delta mediates neurogenic but not mitogenic activation of mitogen-activated protein kinase in neuronal cells. Mol Cell Biol 19: 4209–4218;1999.
7 Csukai M, Chen C-H, De Matteis M-A, Mochly-Rosen D. The coatomer protein β′-COP, a selective binding protein (RACK) for protein kinase Cε. J Biol Chem 272:29200–29206; 1997.
8 Diamond I, Gordon AS. The role of adenosine in mediating cellular and molecular responses to ethanol. EXS 71:175–183;1994.
9 Diamond I, Gordon AS. Cellular and molecular neuroscience of alcoholism. Physiol Rev 77: 1–20;1997.
10 Freund RK, Palmer MR. 8-Bromo-cAMP mimics β-adrenergic sensitization of GABA responses to ethanol in cerebellar Purkinje neurons in vivo. Alcohol Clin Exp Res 20:408–412;1996.
11 Gerstin EH Jr, McMahon T, Dadgar J, Messing RO. Protein kinase C delta mediates ethanol-induced up-regulation of L-type calcium channels. J Biol Chem 273:16409–16414; 1998.
12 Gordon AS, Yao L, Wu ZL, Coe IR, Diamond I. Ethanol alters the subcellular localization of delta- and epsilon protein kinase C in NG108-15 cells. Mol Pharmacol 52:554–559;1997.
13 Gschwendt M, Kittstein W, Marks F. Elongation factor-2 kinase: Effective inhibition by the novel protein kinase inhibitor rottlerin and relative insensitivity towards staurosporine. FEBS Lett 338:85–88;1994.
14 Gschwendt MS, Dieterich J, Rennecke J, Kittstein W, Mueller HJ, Johannes FJ. Inhibition of protein kinase C mu by various inhibitors. FEBS Lett 392:77–80;1996.
15 Harry A, Chen Y, Magnusson R, Iyengar R, Weng G. Differential regulation of adenylyl cyclases by $G\alpha_s$. J Biol Chem 272:19017–19021;1997.
16 Hellevuo K, Yoshimura M, Kao M, Hoffman PL, Cooper DMF, Tabakoff B. A novel adenylyl cyclase sequence cloned from the human erythroleukemia cell line. Biochem Biophys Res Commun 192:311–318;1993.
17 Hellevuo K, Yoshimura M, Mons N, Hoffman PL, Cooper DMF, Tabakoff B. The characterization of a novel human adenylyl cyclase which is present in brain and other tissues. J Biol Chem 270:11581–11589;1995.
18 Hodge CW, Mehmert KK, Kelley SP, McMahon T, Haywood A, Olive MF, Wang D, Sanchez-Perez AM, Messing RO. Supersensitivity to allosteric GABA(A) receptor modulators and alcohol in mice lacking PKC epsilon. Nat Neurosci 2:997–1002;1999.
19 Hoffman PL, Tabakoff B. Ethanol and guanine nucleotide binding proteins: A selective interaction. FASEB J 4:2612–2622;1990.
20 Hornia A, Lu Z, Sukezanc T, Zhong M, Joseph T, Frankel P, Foster DA. Antagonistic effects of protein kinase C alpha and delta on both transformation and phospholipase D activity mediated by the epidermal growth factor receptor. Mol Cell Biol 19:7672–7680;1999.
21 Hundle B, McMahon T, Dadgar J, Chen CH, Mochly-Rosen D, Messing RO. An inhibitory fragment derived from protein kinase C epsilon prevents enhancement of nerve growth factor responses by ethanol and phorbol esters. J Biol Chem 272:15028–15035;1997.
22 Jacobowitz O, Iyengar R. Phorbol ester-induced stimulation and phosphorylation of adenylyl cyclase 2. Proc Natl Acad Sci USA 91: 10630–10634;1994.
23 Johnson JA, Gray MO, Chen CH, Mochly-Rosen D. A protein kinase C translocation inhibitor as an isozyme-selective antagonist of cardiac function. J Biol Chem 271:24962–24966; 1996.

24 Krupinski J, Cali JJ. Molecular diversity of the adenylyl cyclases. In: Cooper DMF, ed. Advances in Second Messenger and Phosphoprotein Research. Philadelphia, Lippincott-Raven, 53–79;1998.

25a Lai HL, Yang TH, Messing RO, Ching YH, Lin SC, Chern Y. Protein kinase C inhibits adenylyl cyclase type VI activity during desensitization of the $A_2$a-adenosine receptor-mediated cAMP response. J. Biol Chem 272:4970–4977; 1997.

25b Li W, Yu JC, Shin DY, Pierce JH. Characterization of a protein kinase C-delta (PKC-delta) ATP binding mutant. J Biol Chem 270:8311–8318;1995.

26 Lin AM, Freund RK, Hoffer BJ, Palmer MR. Ethanol-induced depressions of cerebellar Purkinje neurons are potentiated by β-adrenergic mechanisms in rat brain. J Pharmacol Exp Ther 271:1175–1180;1994.

27 Lin WW, Chang SH, Wang SM. Roles of atypical protein kinase C in lysophosphatidic acid-induced type II adenylyl cyclase activation in RAW 264 macrophages. Br J Pharmacol 128: \\89–1198;1999.

28 Lin WW, Chen BC. Distinct PKC isoforms mediate the activation of cPLA2 and adenylyl cyclase by phorbol ester in RAW264 macrophages. Br J Pharmacol 125:1601–1609;1998.

29 Lu Z, Hornia A, Jiang YW, Zang Q, Ohno S, Foster DA. Tumor promotion by depleting cells of protein kinase C delta. Mol Cell Biol 17: 3418–3428;1997.

30 Luthin GR, Tabakoff B. Activation of adenylate cyclase by alcohols requires the nucleotide-binding protein. J Pharmacol Exp Ther 228: 579–587;1984.

31 Machu TK, Olsen RW, Browning MD. Ethanol has no effect on cAMP-dependent protein kinase-, protein kinase C-, or Ca(2+)-calmodulin-dependent protein kinase II-stimulated phosphorylation of highly purified substrates in vitro. Alcohol Clin Exp Res 15:1040–1044;1991.

32 Martiny-Baron G, Kazanietz MG, Mischak H, Blumberg PM, Kochs G, Hug H, Marme D, Schachtele C. Selective inhibition of protein kinase C isozymes by the indolocarbazole Gö-6976. J Biol Chem 268:9194–9197;1993.

33 McMahon T, Andersen R, Metten P, Crabbe JC, Messing RO. Protein kinase C epsilon mediates up-regulation of N-type calcium channels by ethanol. Mol Pharmacol 57:53–58; 2000.

34 Messing RO, Petersen PJ, Henrich CJ. Chronic ethanol exposure increases levels of protein kinase C delta and epsilon and protein kinase C-mediated phosphorylation in cultured neural cells. J Biol Chem 266:23428–23432;1991.

35 Miyamae M, Rodriguez MM, Camacho SA, Diamond I, Mochly-Rosen D, Figueredo VM. Activation of epsilon protein kinase C correlates with a cardioprotective effect of regular ethanol consumption. Proc Natl Acad Sci USA 95:8262–8267;1998.

36 Mochly-Rosen D. Localization of protein kinases by anchoring proteins: A theme in signal transduction. Science 268:247–251;1995.

37 Mochly-Rosen D, Gordon AS. Anchoring proteins for protein kinase C: A means for isozyme selectivity. FASEB J 12:35–42;1998.

38 Mons N, Cooper DMF. Adenylate cyclases: Critical foci in neuronal signaling. Trends Neurosci 18:536–542;1995.

39 Moore MS, DeZazzo J, Luk AY, Tully T, Singh CM, Heberlein U. Ethanol intoxication in *Drosophila*: Genetic and pharmacological evidence for regulation by the cAMP signaling pathway. Cell 93:997–1007;1998.

40 Nishikawa K, Toker A, Johannes F-J, Songyang Z, Cantley LC. Determination of the specific substrate sequence motifs of protein kinase C isozymes. J Biol Chem 272:952–960; 1997.

41 Nishizuka Y. Protein kinase C and lipid signaling for sustained cell responses. FASEB J 9: 484–496;1995.

42 Parmer TG, Ward MD, Hait WN. Effects of rottlerin, an inhibitor of calmodulin-dependent protein kinase III, on cellular proliferation, viability, and cell cycle distribution in malignant glioma cells. Cell Growth Differ 8:327–334; 1997.

43 Patrone M, Pessino A, Passalacqua M, Sparatore B, Milloni E, Ponremoli S. Correlation between levels of delta protein kinase C and resistance to differentiation in murine erythroleukemia cells. Biochem Biophys Res Commun 220:26–30;1996.

44 Pongracz J, Webb P, Wang K, Deacon E, Lunn OJ, Lord JM. Spontaneous neutrophil apoptosis involves caspase 3-mediated activation of protein kinase C-delta. J Biol Chem 274: 37329–37334;1999.

45 Premont RT. Identification of adenylyl cyclases by amplification using degenerate primers. Methods Enzymol 238:116–127;1994.

46 Rabbani M, Nelson E, Hoffman PL, Tabakoff B. Role of protein kinase C in ethanol-induced activation of adenylyl cyclase. Alcohol Clin Exp Res 23:77–86;1999.

47 Reyland ME, Anderson SM, Matassa AA, Barzen KA, Quissel DO. Protein kinase C delta is essential for etoposide-induced apoptosis in salivary gland acinar cells. J Biol Chem 274: 19115–19123;1999.

48 Ron D, Chen CH, Caldwell J, Jamieson L, Orr E, Mochly-Rosen D. Cloning of an intracellular receptor for protein kinase C: A homolog of the beta subunit of G proteins. Proc Natl Acad Sci USA 91:839–843;1994.

49 Ron D, Luo J, Mochly-Rosen D. C2 region-derived peptides inhibit translocation and function of β protein kinase C in vivo. J Biol Chem 270:24180–24187;1995.

50 Saito T, Lee JM, Tabakoff B. Ethanol's effects on cortical adenylate cyclase activity. J Neurochem 44:1037–1044;1985.

51 Smit MJ, Iyengar R. Mammalian adenylyl cyclases. In: Cooper DMF, ed. Advances in Second Messenger and Phosphoprotein Research. Philadelphia, Lippincott-Raven, 1–21;1998.

52 Sunahara RK, Dessauer CW, Gilman AG. Complexity and diversity of mammalian adenylyl cyclases. Annu Rev Pharmacol Toxicol 36:461–480;1996.

53 Tabakoff B, Hoffman PL. Adenylyl cyclases and alcohol. In: Cooper DMF, ed. Advances in Second Messenger and Phosphoprotein Research. Philadelphia, Lippincott-Raven, 173–193;1998.

54 Tang WJ, Hurley JH. Catalytic mechanism and regulation of mammalian adenylyl cyclases. Mol Pharmacol 54:461–480;1998.

55 Taussig R, Zimmermann G. Type-specific regulation of mammalian adenylyl cyclases by G proteins. In: Cooper DMF, ed. Advances in Second Messenger and Phosphoprotein Research. Philadelphia, Lippincott-Raven, 81–98;1998.

56 Tesmer JJG, Sunahara RK, Gilman AG, Sprang SR. Crystal structure of the catalytic domains of adenylyl cyclase in a complex with Gsα·GTPγS. Science 278:1907–1916;1997.

57 Tsunoda S, Sierralta J, Sun Y, Bodner R, Suzuki E, Becker A, Socolich M, Suker CS. A multivalent PDZ-domain protein assembles signalling complexes in a G-protein-coupled cascade. Nature 388:243–249;1997.

58 Villalba M, Kasibhatla S, Genestier L, Mahboubi A, Green DR, Altman A. Protein kinase C theta cooperates with calcineurin to induce Fas ligand expression during activation-induced T cell death. J Immunol 163:5813–5819; 1999.

59 Watson PA, Krupinski J, Kempinski AM, Frankenfield CD. Molecular cloning and characterization of the type VII isoform of mammalian adenylyl cyclase expressed widely in mouse tissues and in S49 mouse lymphoma cells. J Biol Chem 269:28893–28898;1994.

60 Yang X, Diehl AM, Wand GS. Ethanol exposure alters the phosphorylation of cyclic AMP responsive element binding protein and cyclic AMP responsive element binding activity in rat cerebellum. J Pharmacol Exp Ther 278: 338–346;1996.

61 Yang X, Horn K, Wand GS. Chronic ethanol exposure impairs phosphorylation of CREB and CRE-binding activity in rat striatum. Alcohol Clin Exp Res 22:382–390;1998.

62 Yoshimura M Cooper DMF. Type-specific stimulation of adenylyl cyclase by protein kinase C. J Biol Chem 268:4604–4607;1993.

63 Yoshimura M, Ikeda H, Tabakoff B. μ-Opioid receptors inhibit dopamine-stimulated activity of type V adenylyl cyclase but enhance dopamine-stimulated activity of type VII adenylyl cyclase. Mol Pharmacol 50:43–51;1996.

64 Yoshimura M, Tabakoff B. Selective effects of ethanol on the generation of cAMP by particular members of the adenylyl cyclase family. Alcohol Clin Exp Res 19:1435–1440;1995.

65 Yoshimura M, Tabakoff B. Ethanol's actions on cAMP-mediated signaling in cells transfected with type VII adenylyl cyclase. Alcohol Clin Exp Res 23:1457–1461;1999.

66 Zhang G, Liu Y, Ruoho AE, Hurley JH. Structure of the adenylyl cyclase catalytic core. Nature 386:247–253;1997.

67 Zimmermann G, Taussig R. Protein kinase C alters the responsiveness of adenylyl cyclases to G protein α and βγ subunits. J Biol Chem 271: 27161–27166;1996.