Ethanol-induced Translocation of cAMP-dependent Protein Kinase to the Nucleus

MECHANISM AND FUNCTIONAL CONSEQUENCES*

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Ethanol induces translocation of the catalytic subunit (Ca) of cAMP-dependent protein kinase (PKA) from the Golgi area to the nucleus in NG108–15 cells. Ethanol also induces translocation of the RIβ regulatory subunit of PKA to the nucleus, RI and Ca are not translocated. Nuclear PKA activity in ethanol-treated cells is no longer regulated by cAMP. Gel filtration and immunoprecipitation analysis confirm that ethanol blocks the reassociation of Ca with RI but does not induce dissociation of these subunits. Ethanol also reduces inhibition of Ca by the PKA inhibitor PKI. Pre-incubation of Ca with ethanol decreases phosphorylation of Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide) and casein but has no effect on the phosphorylation of highly charged molecules such as histone H1 or protamine. cAMP-response element-binding protein (CREB) phosphorylation by Ca is also increased in ethanol-treated cells. This increase in CREB phosphorylation is inhibited by the PKA antagonist (Rα)-cAMPS and by an adenosine receptor antagonist. These results suggest that ethanol affects a cascade of events allowing for sustained nuclear localization of Ca and prolonged CREB phosphorylation. These events may account for ethanol-induced changes in cAMP-dependent gene expression.

The cAMP signaling pathway is a major target for ethanol in intact cells (1). Receptor-stimulated increases in intracellular cAMP levels cause dissociation of the catalytic subunit of cAMP-dependent protein kinase (PKA)1 from regulatory subunits; the free catalytic subunit (Ca) then phosphorylates intracellular substrates. The duration of the stimulus determines whether these substrates are cytoplasmic or nuclear (2). In the nucleus, PKA Ca phosphorylates a specific transcription factor, CREB, at serine 133, initiating changes in expression of genes containing cAMP response elements (3–5). Translocation of Ca to the nucleus following receptor activation is transient, and Ca rapidly exits the nucleus (6). Export of Ca out of the nucleus is mediated by binding of Ca to the heat stable PKA inhibitor, PKI, which contains a nuclear export signal (7, 8).

Brief exposure to ethanol increases basal (9, 10) and receptor-stimulated cAMP production in neuronal cells in culture and isolated brain preparations (9–13). In rat cerebellum, acute ethanol exposure causes an increase in both phosphorylated CREB and cAMP-responsive element binding activity (14). Chronic exposure to ethanol results in an adaptive desensitization of cAMP production, in cells in culture (9, 15–17), lymphocytes isolated from human alcoholics (18), and in mouse brains (19). There is also a decrease in the phosphorylation of CREB in granule cells of rat cerebellum (20). Decreased cAMP production may be because of a decrease in the amount and/or activity of several key proteins in this pathway, including Gα (21), Gβ (22), PKA regulatory subunit RI (23), and PKA catalytic activity (24, 25). Increases in Gα have also been reported after chronic exposure to ethanol (26).

Recently, we have shown that one of the catalytic subunits of PKA, Ca, translocates to the nucleus of NG108–15 cells during prolonged exposure to ethanol; Ca remains in the nucleus as long as ethanol is present (23). This is in contrast to the rapid exit of Ca from the nucleus when adenyl cyclase and PKA are activated by receptors or forskolin (6). Because prolonged intranuclear localization of Ca during exposure to ethanol could be responsible for ethanol-induced changes in gene expression (see Ref. 1 for review), we undertook a study to determine the mechanism and functional significance of ethanol-induced Ca translocation. We show here that ethanol-induced nuclear Ca is functionally active, resulting in the phosphorylation of CREB. This persistent activation appears to be because of, in part, ethanol inhibition of Ca reassociation with regulatory subunits of PKA and ethanol-dependent inactivation of PKI.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were purchased from Sigma except where indicated.

Cell Culture—NG108–15 neuroblastoma × glioma hybrid cells obtained from the cell culture facility at the University of California (San Francisco, CA) were grown in 10% Serum Plus (JRH Biosciences, Lenexa, KS) in a 10% CO2 incubator at 37 °C, as described previously (17). The cells were seeded at a density of 2 × 105 cells/ml, then incubated for 4 hr before exposure to ethanol. Cells were cultured in complete defined medium (10). On day 3, media were changed, and cells were maintained in the absence or presence of 200 mM ethanol for 24 h; media were replaced daily. Treatment with 10 μM forskolin was carried out for 30 min. 40 μM (Rα) cAMPS (Biolog Life Science Institute, La Jolla, CA) and 10 μM BWA1434U (a gift from Glaxo Wellcome) were added 3 hr or 30 min, respectively, prior to ethanol or forskolin exposure.

Immunocytochemistry—Cells were fixed, blocked, and incubated with primary and secondary antibodies as described elsewhere (23). Antibody against CREB phosphorylated at Ser-133 was purchased from NEN BioLabs (Beverly, MA) and diluted 1:200. Secondary antibody (fluorescein isothiocyanate-conjugated goat anti-rabbit) was purchased...
from Cappel (Costa Mesa, CA) and diluted 1:250. 

**Nuclear Extracts**—Nuclei were isolated from NG108–15 cells in hypertonic sucrose by the procedure of Laks et al. (27). The nuclear pellet was resuspended in extraction buffer (10 mM Tris-HCl, pH 7.4, 3 mM MgCl₂, 2 mM dithiothreitol, 0.5 mM NaCl, 0.1 mM EDTA, protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 100 µg/ml aprotinin, 100 µg/ml leupeptin), and phosphatase inhibitors (20 mM NaF, 1 mM sodium orthovanadate)) and shaken at 4 °C for 60 min. After centrifugation for 20 min at 67,000 × g, protein content and PKA activity were determined in the supernatant, and a fraction was used for Western blot analysis.

**PKA Assay**—Nuclear extracts (0.6 mg/ml protein) were incubated in 50 mM phosphate buffer in the presence and absence of 5 µM cAMP. The reaction was started by adding 10 µl of reaction mixture containing 10 mM magnesium acetate, 200 µM ATP, 200 µM Kemptide, and 12.5 µCi/ml [γ-32P]ATP, specific activity 3000 Ci/mmol (Amersham Pharmacia Biotech). The reaction was stopped after incubation for 5 min at 37 °C, and the amount of γ-32P incorporated into Kemptide was determined as described by Rabin et al. (11). The PKA catalytic subunit (Sigma) was reconstituted in 40 mM dithiothreitol to a final concentration of 0.05 mg/ml and was used within 3 days. For each experiment, enzyme was diluted 50-fold in assay buffer with or without various concentrations of ethanol. The final concentration of enzyme was 0.51 units/0.13 mM histone H1 (Life Technologies, Inc.) and 0.2 mM Kemptide, casein, or protamine sulfate.

**Gel Filtration Assay**—A Sephadex G-75 column (Amersham Pharmacia Biotech) (15 × 250 mm) was preequilibrated with binding buffer (50 mM potassium phosphate, pH 7.0, 4 mM 2-mercaptoethanol, and 75 mM NaCl) (28) and used to separate PKA holoenzyme from dissociated Ca⁺ and RII subunits. The void volume was determined to be 14 ml. At a flow rate of 1 ml/min, histone eluted in the void volume (RII at 36 ml and Ca at 40 ml). Kinase activity was measured in these fractions as described above, using 10 mg/ml protamine sulfate as substrate, because ethanol has no effect on phosphorylation of this substrate (see Fig. 4B). Phosphorylation in the void volume containing histone is cAMP-dependent, whereas kinase activity in the 40-ml fraction containing Ca is cAMP-independent.

**Immunoprecipitation**—Nuclear extract (0.5 mg of protein) was prepared as described above (except that NaCl was omitted from extraction buffer) and was precleared for 30 min with protein A-conjugated agarose. Supernatant from a 10 min centrifugation at 10,000 rpm was incubated overnight with 2 µg of polyclonal Ca antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and further incubated with protein A-conjugated agarose for 1 h. The immunoprecipitate was centrifuged and washed four times with phosphate-buffered saline, pH 7.0. All incubations were at 4 °C. After a final wash, the immunoprecipitate was resuspended in SDS sample buffer, subjected to SDS-polyacrylamide gel electrophoresis, and probed with RII antibodies (Transduction Laboratories, Lexington, KY) and Ca antibodies on Western blots.

**Western Blots**—Whole cell lysates were prepared by washing cells with ice-cold phosphate-buffered saline and collecting them in SDS buffer (100 µl well from 4 wells) followed by sonication for 3 s. Cells from 2 remaining wells were used for protein measurements. Nuclear extracts and cell lysates were subjected to SDS-polyacrylamide gel electrophoresis (29), and proteins were transferred to polyvinylidene difluoride membranes. Blots were probed using standard procedures (30) and antibodies against Ca (a generous gift from Susan Taylor, University of California, San Diego, CA) (diluted 1:10,000), RI, RIIa, and RIIb (Transduction Laboratories) (1:1,000), Cβ (Santa Cruz Biotechnology Inc.) (1:1,000), or total CREB and phosphorylated CREB (NEN BioLabs) (1:1,000). Secondary antibodies (NEN BioLabs) were horseradish peroxidase-linked goat anti-rabbit (1:5,000) for Ca, Cβ, CREB, and phospho-CREB and rabbit anti-mouse (1:10,000) for RI and RII. Proteins were detected using LumiGLO chemiluminescence substrate (NEN BioLabs) and exposed to Kodak Biomax film. Scanning densitometry was used to quantitate Western blots using the NIH Image program.

**Results**

**Ethanol Increases the Amount of Ca and RIIb in the Nucleus of NG108–15 Cells**—Using immunocytochemistry, we have shown that chronic ethanol exposure causes translocation of PKA Ca from the Golgi area into the nucleus in NG108–15 cells (23). To quantitate the extent of Ca translocation and to determine whether ethanol causes translocation to the nucleus of other PKA subunits in NG108–15 cells, we carried out Western blots on cell lysates and nuclei from these cells. Fig. 1 shows that NG108–15 cells express Ca, Cβ, RI, and RIIb. Only traces of RIIa could be detected in the presence or absence of ethanol (data not shown). After incubation with ethanol for 24 h, there is a 54 ± 2% (mean ± S.E.) increase in the amount of Ca in the nuclei of ethanol-treated cells and a 40 ± 5% decrease in the amount of RI in whole cell homogenates. These results are in agreement with our previous findings (23) that ethanol causes translocation of Ca into the nucleus and decreases the amount of RI subunit in whole cells. Fig. 1 also shows that, after exposure to ethanol, nuclear RIIb increases by 52 ± 4%. However, there is no RI or Cβ in the nuclei of either control or ethanol-treated cells.

**Nuclear PKA Activity is cAMP-independent in Ethanol-treated NG108–15 Cells**—Our data suggest that the amount of Ca and RIIb in the nucleus of NG108–15 cells increases by 50% after chronic exposure to ethanol. To determine whether nuclear Ca is active or bound to RIIb and thus inactive, we measured PKA activity in the presence and absence of cAMP in nuclear extracts. Nuclei were recovered after cell disruption and centrifugation in hypertonic sucrose buffer, and soluble proteins were extracted with 0.5 M NaCl buffer (27). PKA activity in the extract was assayed with Kemptide as substrate (11). Fig. 2 shows that PKA activity remaining in control nuclei after subcellular fractionation is cAMP-dependent, suggesting that Ca and RIIb are associated as a holoenzyme. In contrast, kinase activity in the nuclei of cells treated with 200 mM ethanol for 24 h is cAMP-independent, suggesting that some of the Ca is no longer associated with RIIb. Because total PKA activity did not change in ethanol-treated cells, it is also likely that a significant fraction of nuclear PKA is inactive after ethanol exposure.

**Ethanol Inhibits Binding of Ca to RII**—The results in Fig. 2 suggest that active Ca is not regulated by RII in the presence of ethanol. To test this possibility, we examined RII inhibition of purified Ca activity in the presence or absence of ethanol. Fig. 3A shows that RII inhibits Ca catalytic activity by 72 ± 2% in the absence of ethanol. However, preincubation of RII with ethanol strikingly decreases RII inhibition of Ca. When RII was preincubated with 50 mM ethanol for 2 h, inhibition of Ca activity by RII was reduced to 25 ± 1%. These results suggest that ethanol inhibits the binding of RII to Ca, thereby preventing RII inhibition of Ca activity.

To determine directly whether ethanol inhibits binding of RII to Ca, association of the two subunits was assessed by Sephadex G-75 gel filtration chromatography. The holoenzyme

![Fig. 1. Immunodetection of PKA subunits in NG108–15 cells.](image-url)
Elution of PKA subunits—Since PKI inhibits the reassociation of PKA subunits of PKA by PKI, suggesting that ethanol, similar to its effect on RII, probably inhibits the binding of PKI to Ca. This would also prevent the exit of Ca from the nucleus.

Ethanol Inhibits Interaction of PKI with CcAMP—Because PKI and RII share common binding sites on CcAMP with substrates (31), we determined the effect of ethanol on Cα-mediated phosphorylation of different substrates in vitro. Fig. 4B shows that Cα preincubation with 100 mM ethanol for 30 min inhibits Cα-dependent phosphorylation of Kemptide or casein by 36%. However, 100 mM ethanol did not inhibit phosphorylation of the highly charged molecules histone H1 or protamine sulfate. Similar results were obtained with 50 mM ethanol. Moreover, preincubation of all substrates with 100 mM ethanol did not affect subsequent phosphorylation, except for casein where inhibition of phosphorylation was reduced from 36 to 25% (data not shown). These results suggest that ethanol inhibits phosphorylation of hydrophobic substrates by interacting primarily with hydrophobic sites on Ca.

Ethanol-induced Translocation of Cα Leads to Phosphorylation of CREB—Cα localized to the nucleus after exposure to ethanol is active (Fig. 2), suggesting that endogenous nuclear substrates should also be phosphorylated. The transcription factor CREB is a physiological substrate for PKA in the nucleus (3–5). Therefore, we used Western blot analysis and immunocytochemistry with polyclonal antibodies that recognize CREB phosphorylated at serine 133 (phospho-CREB) to determine whether ethanol increases CREB phosphorylation. Fig. 5, A and B, shows that ethanol induces phosphorylation of CREB (p-CREB) reaching a maximum at 3 h of exposure. Most importantly phospho-CREB remains higher than control levels from 1–24 h of ethanol treatment; there was no change in the amount of CREB (Fig. 5A). Because CREB can be phosphorylated at serine 133 by several other kinases (32–37), we used the PKA antagonist (R<sub>g</sub>)-cAMPS to confirm that the ethanol-induced increase in phospho-CREB was due primarily to PKA. Fig. 6 shows an immunocytochemical image of phospho-CREB in NG108–15 cells before and after treatment with either forskolin or ethanol. The amount of phospho-CREB in the nucleus after a 3-h exposure to ethanol is similar to that obtained by treating the cells with forskolin for 30 min (Fig. 6). Forskolin- and ethanol-induced phosphorylation of CREB was greatly reduced by preincubating cells with (R<sub>g</sub>)-cAMPS, suggesting that PKA is responsible for CREB phosphorylation induced by ethanol.

Adenosine A2 Receptors Mediate Ethanol-induced CREB Phosphorylation—We have previously shown that adenosine A2 receptors mediate acute ethanol-induced increases in cAMP levels; adenosine A<sub>2</sub> receptor blockade completely prevents ethanol-induced cAMP accumulation (9, 10). If adenosine receptor-dependent increases in cAMP levels are responsible for ethanol-induced activation and translocation of Cα to the nucleus, an adenosine receptor antagonist should also block phosphorylation of CREB. BWA1434U, an adenosine receptor antagonist, blocked ethanol-induced CREB phosphorylation but, as expected, had no effect on forskolin-induced CREB phosphorylation, which bypasses receptor stimulation (Fig. 6).

**DISCUSSION**

Immunocytochemical studies in our laboratory have shown that chronic exposure of NG108–15 cells to ethanol caused a dramatic and specific translocation of Ca from the Golgi area to...
the nucleus (23). This translocation is confirmed by Western blot analysis of homogenates and nuclei of control and ethanol-treated cells (Fig. 1). We demonstrate here that the RII regulatory subunit is also translocated to the nucleus following chronic ethanol exposure, but ethanol does not alter localization of the Cα catalytic subunit. Nuclei of control NG108–15 cells contain both Cα and RIIβ, probably associated as holoenzyme, because cAMP is required for PKA activation (Fig. 2). After chronic ethanol exposure, however, there is a substantial increase in the amounts of both Cα and RIIβ in the nucleus (Fig. 1). Once in the nuclei of ethanol-treated cells, some Cα and RIIβ appear to be dissociated because PKA activity is cAMP-independent (Fig. 2). This is likely because of the inhibition of the association of Cα and RII by ethanol (Fig. 3B) thereby blocking RII regulation of Cα activity (Fig. 3A). Thus, a 90-min incubation with 50 mM ethanol prevents the association of RII and Cα by 50% (Fig. 3B) and decreases RII inhibition of Cα activity by 45% (Fig. 3A). This concentration of ethanol is clinically relevant, particularly in chronic alcoholics (38). Moreover, ethanol inhibition of Cα:RIIβ holoenzyme formation in vitro is reproduced in vivo because 45% of nuclear RIIβ fails to co-immunoprecipitate with Cα after 24 h of ethanol treatment (Fig. 3D).

Our data also suggest a mechanism for the prolonged seques-
Ethanol activates PKA in the nucleus.

Functionally active Ca in the nucleus in the presence of ethanol. Neurotransmitter- and hormone-dependent signal transduction via cAMP cause a transient translocation of PKA Ca from the cytoplasm to the nucleus with brief activation of transcription; Ca returns rapidly to the cytoplasm after binding to PKI (6–8). During prolonged exposure to ethanol, however, Ca remains in the nucleus and returns to the Golgi only 6–12 h after ethanol is removed (23). This unusual time course for Ca translocation could be because of the interaction of ethanol with hydrophobic binding sites on PKI that prevents PKI from binding to Ca, thereby inhibiting PKI-mediated export of Ca out of the nucleus. Indeed, the data presented here show that PKI inhibition of Ca activity is greatly reduced after preincubation with ethanol (Fig. 4A). Presumably this is because ethanol prevents the binding of PKI to Ca, similar to ethanol interfering with the association of RIIβ with Ca (Fig. 3, B and D). Ethanol also appears to limit access of specific substrates to Ca; phosphorylation of Kemptide and casein by Ca is reduced after preincubation of Ca with ethanol, whereas phosphorylation of histone and protamine are unaffected by ethanol (Fig. 4B). The binding of substrates and RII and PKI to the catalytic subunit of PKA involves two hydrophobic interactions and three electrostatic contacts (31). Recent evidence suggests that ethanol can bind directly to hydrophobic pockets in proteins (39–41). Therefore, we propose that ethanol-induced sustained translocation of Ca to the nucleus is related to ethanol inhibition of the reassociation of Ca with RII and/or the binding of Ca to PKI. This may be because of ethanol-induced conformational changes at hydrophobic sites near the binding domains. This explanation is also consistent with our observation that ethanol does not inhibit phosphorylation of substrates that are highly charged, such as histone H1 and protamine sulfate (Prot) (Fig. 4B). We presume this occurs because Kemptide and casein have a random coil secondary structure (42, 43) that is less affected by ethanol.

Two apparent methodological discrepancies in our studies are informative. First, in our previous paper (23), ethanol caused the translocation to the nucleus of almost all Ca observable by confocal microscopy. In this report, Western blot anal-
Ethanol activates PKA in the nucleus

Fig. 6. Ethanol-induced CREB phosphorylation in NG108–15 cells is blocked by adenosine receptor and PKA antagonists. Immunocytochemical detection of phospho-CREB in NG108–15 cells treated with ethanol for 3 h. Positive control: cells treated for 30 min with 10 μM forskolin. Pretreatment with (R<sub>p</sub>)-cAMPS and BWA1434U was as described under “Experimental Procedures.” All images are ×400 magnification obtained with a Bio-Rad 1024 confocal microscope. Data shown are representative of three experiments.

Analysis indicates that only approximately 50% of Ca<sub>a</sub> is translocated to the nucleus. This discrepancy can be explained by methodological differences; we used confocal microscopy in our earlier report and Western blot analysis in this study. Confocal microscopy results in visualization of densely localized Ca<sub>a</sub>, as seen in the Golgi and nuclei. Diffusely distributed Ca<sub>a</sub>, on the other hand, is more difficult to observe by microscopy but is readily detected by Western blot analysis. The second discrepancy is that Western blot analysis shows a 50% increase in Ca<sub>a</sub> in the nucleus after exposure to ethanol; this is not accompanied by an increase in PKA enzymatic activity, suggesting that some nuclear Ca<sub>a</sub> may be rendered inactive and no longer regulated by cAMP after chronic exposure to ethanol. This raises the very interesting possibility that some Ca<sub>a</sub> may be retained in the nucleus after chronic exposure to ethanol by binding to a putative inactivating protein distinct from PKI and RII. The existence of such a hypothetical Ca<sub>a</sub>-binding protein in the nucleus is suggested by our observation that sustained nuclear localization of Ca<sub>a</sub> in the presence of ethanol requires protein synthesis. Studies are underway to identify this putative binding protein.

Ethanol-induced translocation of Ca<sub>a</sub> to the nucleus should enhance cAMP-dependent gene expression and reduce phosphorylation of membrane or cytoplasmic proteins. Because Ca<sub>a</sub> is active for long periods of time, there may even be inappropriate transcription of cAMP-responsive genes. This is suggested by our finding that ethanol causes a striking increase in CREB phosphorylation with a peak at 3 h, followed by a sustained increase in phospho-CREB even after 24 h of ethanol exposure (Fig. 5). The peak of CREB phosphorylation at 3 h appears to be because of unrestrained Ca<sub>a</sub> activity, apparently because ethanol inhibits RII and PKI binding to Ca<sub>a</sub> (Figs. 3 and 4A). After 3 h, phospho-CREB remains higher than in control cells but decreases despite the continued presence of Ca<sub>a</sub> in the nucleus (see Ref. 18 and Fig. 4). This may be related to the observation that CREB phosphorylation following activation of the cAMP pathway is also regulated by protein phosphatases such as protein phosphatase 2B in neurons of the striatum (44) and protein phosphatase 1 in PC12 cells (45). Therefore, the decrease in CREB phosphorylation after 3 h may be because of an ethanol-induced increase in nuclear protein phosphatase activity.

Several reports have shown that other kinases can phosphorylate CREB in vitro and in vivo on Ser-133, e.g. calmodulin-dependent kinase IV (32–35), mitogen-activated protein kinase (36), and protein kinase C (37). In NG108–15 cells, the increase in CREB phosphorylation during ethanol exposure appears to be due primarily to Ca<sub>a</sub>, because the PKA-specific inhibitor (R<sub>p</sub>)-cAMPS, which acts directly on the holoenzyme to prevent its dissociation, significantly but not completely inhibits phosphorylation of CREB (Fig. 6). This suggests the possibility that an early activation of PKA by ethanol-induced increases in cAMP may also up-regulate other kinases that phosphorylate CREB. However, an ethanol-induced increase in cAMP production must be required for CREB phosphorylation in the nucleus because BWA1434U, an adenosine receptor antagonist that blocks acute ethanol-induced increases in cAMP levels (9, 10), completely abolishes ethanol-induced phosphorylation of CREB (Fig. 6). This is consistent with recent data in our laboratory showing that both (R<sub>p</sub>)-cAMPS and BWA1434U prevent acute ethanol-induced translocation of Ca<sub>a</sub> into the nucleus. Moreover, a requirement for cAMP is also supported by data in Fig. 3C showing that ethanol alone does not dissociate the PKA holoenzyme.

In summary, the major finding in this paper is that ethanol-induced translocation of PKA Ca<sub>a</sub> to the nucleus in NG108–15 cells causes a sustained increase in CREB phosphorylation. CREB phosphorylation has long been implicated in the regulation of many cellular functions and in short and long term learning and memory (46–48). Taken together with recent genetic data implicating cAMP signaling in alcohol-related behaviors (49, 50), ethanol regulation of cAMP-dependent gene transcription may play an important role in molecular mechanisms that underlie alcoholism and addictive behaviors.

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