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ACCELERATED COMMUNICATION

Ethanol Alters the Subcellular Localization of δ- and ϵ Protein Kinase C in NG108–15 Cells

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SUMMARY

Protein kinase C (PKC) has been shown to regulate the ethanol sensitivity of membrane-bound receptors and transporters, but little is known about the molecular mechanisms underlying this regulation. PKC is a family of isozymes that translocate to new intracellular sites on activation. Here we present immunochemical data showing that ethanol causes translocation of δ- and ϵ-PKC to new intracellular sites. Ethanol causes translocation of δ-PKC from the Golgi to the perinucleus; this translocation is similar to that induced by activation of PKC with phorbol esters. In contrast, ϵ-PKC translocation caused by ethanol is different from that induced by phorbol esters; ethanol causes translocation of ϵ-PKC from the perinucleus to the cytoplasm, whereas phorbol ester activation causes translocation of ϵ-PKC to the nucleus. Because the substrate specificity of these kinases is determined by their site of localization, ethanol-induced translocation of δ- and ϵ-PKC to new intracellular sites may explain some of the pleiotropic effects of ethanol on cellular functions.

PKC, a family of isozymes that mediates multiple cellular functions, has been shown to regulate the effects of ethanol on receptors (1–6) and membrane-bound transporters (7), but the mechanism underlying this regulation is unknown. Ethanol alters the amount, activity, and subcellular distribution of PKC. Increased amounts of α-, δ-, and ϵ-PKC in NG108–15 cells (8) and of δ- and ϵ-PKC in PC12 cells (9) are found after chronic ethanol exposure, and there is increased activity of PKC in NG108–15 and PC12 cells (9). PKC activity is also increased in human platelets (10), lymphocytes (11), and epidermal keratinocytes (12) after acute ethanol exposure. Moreover, ethanol causes translocation of PKC activity from cytosolic to membrane fractions in astroglial cells (13), human lymphocytes (11), and epidermal keratinocytes (12).

On activation, each PKC isozyme translocates from a specific intracellular site to another (14). Recent evidence suggests that the specificity of substrate phosphorylation of each isozyme is determined by its localization (14). Ethanol-induced activation and translocation of specific PKC isozymes to new intracellular sites could therefore account for many of the pleiotropic effects of ethanol on cell functions. To test this hypothesis, we carried out studies on the localization of δ- and ϵ-PKC in NG108–15 neuroblastoma × glioma hybrid cells. We report here that ethanol causes translocation of δ- and ϵ-PKC to new intracellular sites in these cells.

Materials and Methods

Cell culture. NG108–15 neuroblastoma × glioma hybrid cells were seeded in single-chamber slides in defined media consisting of Dulbecco's modified Eagle's medium/Ham's F-12 medium (3:1); 0.1 mM hypoxanthine; 1.0 μM aminopterin; 12 mM thymidine; 25 mM HEPES, pH 7.4; trace elements (0.5 nM $MnCl_2$, 0.5 nM $[NH_4]_6Mo_7O_{24}$, 0.25 nM $SnCl_4$, 25 nM Na_3VO_4, 5 nM $CdSO_4$, 0.25 nM $NiSO_4$, 15 nM H_2SeO_3, 25 nM Na_2SiO_3); bovine insulin (5 μg/ml); human transferrin (50 μg/ml); and oleic acid (10 μg/ml) complexed with fatty acid-free bovine serum albumin (2 mg/ml) (15) at a density of 3.2×10^3 cells/cm^2 and maintained for 48 hr. Media were then

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ABBREVIATIONS: PKC, protein kinase C; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS, phosphate-buffered saline; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; TBS, Tris-buffered saline; PMA, phorbol 12-myristate 13-acetate; DAG, diacylglycerol; RACK, receptor for activated C kinase; PKA, cAMP-dependent protein kinase; ANOVA, analysis of variance.

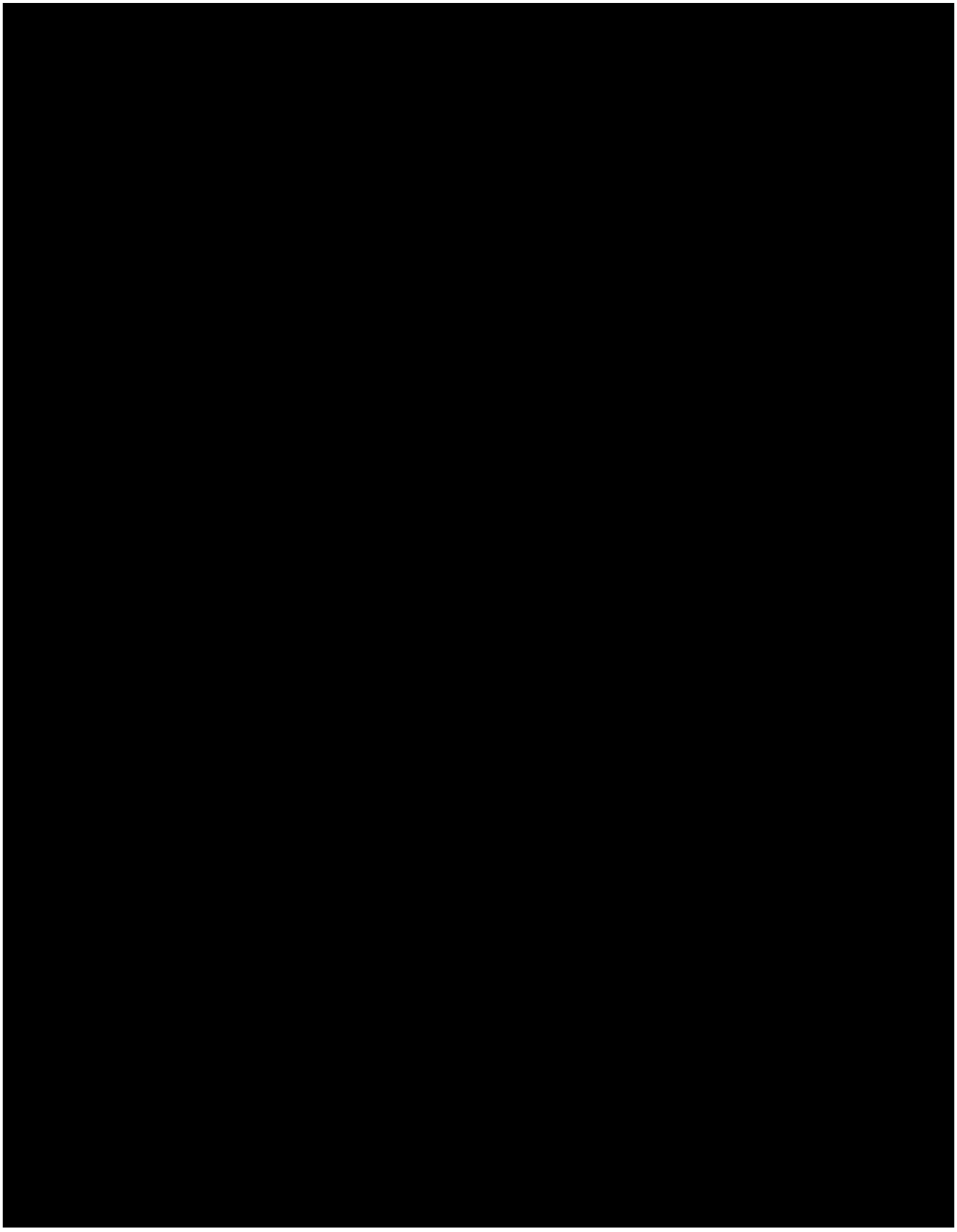

Fig. 1. Ethanol induces reversible translocation of δ- and ϵ-PKC. A, NG108–15 cells were incubated with 200 mM ethanol for the indicated times or with 100 nM β-PMA for 10 min. B, NG108–15 cells were incubated with 200 mM ethanol for 48 hr and then media replaced with fresh media without ethanol as described in Materials and Methods. Cells were fixed and stained for δ- and ϵ-PKC as described in Materials and Methods and scanned using a Bio-Rad 1024 confocal microscope. *Bar,* 25 μm. False color image scale is displayed beneath the images.

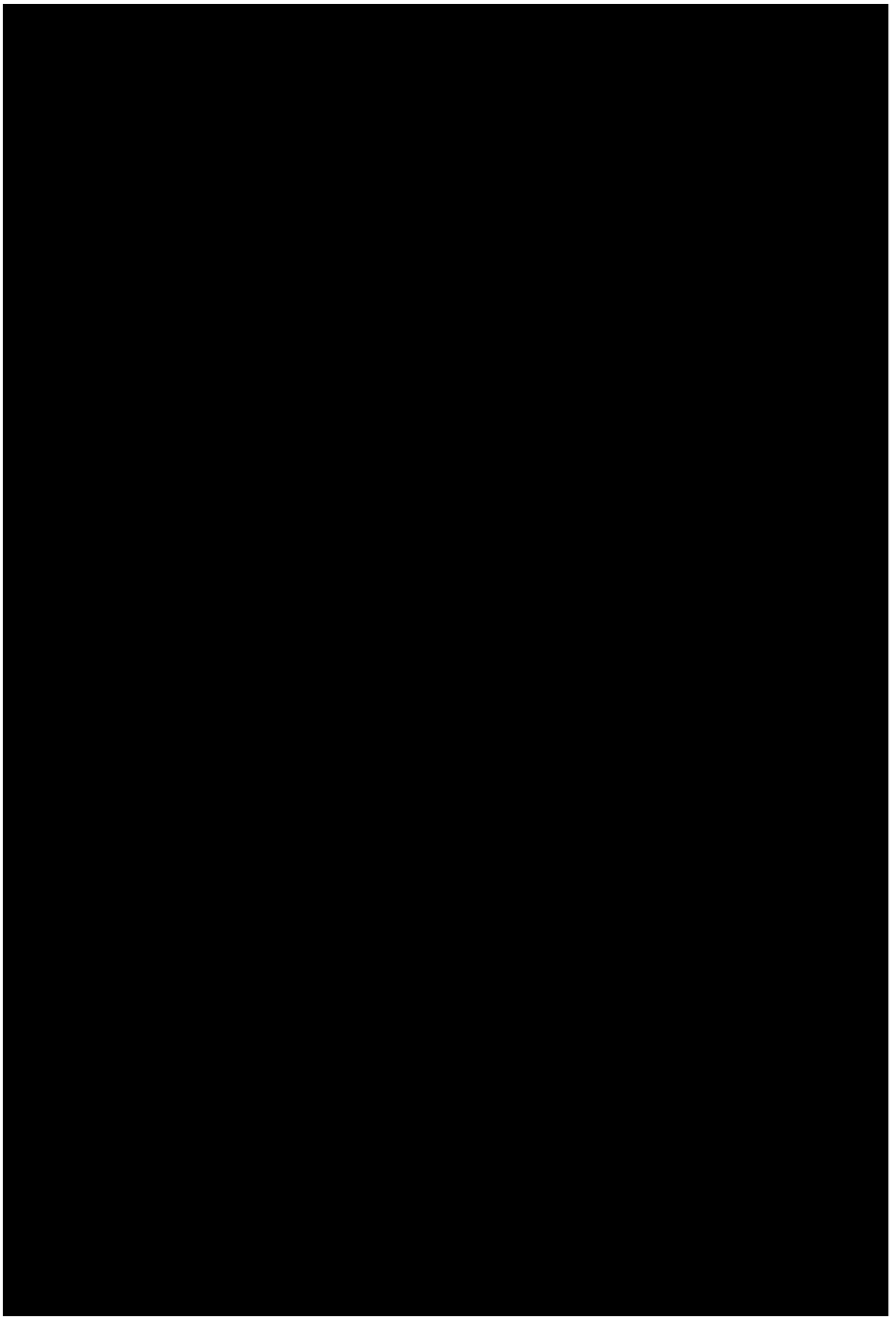

Fig. 1b.

replaced daily by defined media with or without various concentrations of ethanol. Slides were wrapped in parafilm to prevent ethanol evaporation and maintained for the indicated time. For the ethanol withdrawal experiments, NG108–15 cells were incubated with media containing 200 mM ethanol for 48 hr, which was replaced with fresh media without ethanol for 48 hr, with a media change at 24 hr.

Immunocytochemistry. Cells were fixed with methanol (−20°) for 2–3 min. Slides were then rinsed on ice three times for 5 min each in PBS and incubated at room temperature with blocking buffer (1% normal goat serum in PBS, 0.1% Triton X-100) for 3–4 hr, followed by overnight incubation with primary antibody solution at 4° in a humidified chamber. Antibodies against δ- and ϵ-PKC (Santa Cruz Biotechnology, Santa Cruz, CA) were diluted 1:150 and 1:100, respectively, in PBS containing 0.1% Triton X-100 and 2 mg/ml fatty acid-free bovine serum albumin. Slides were then washed as before and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG for 1 hr. The slides were washed again; coverslips were affixed using Vectashield mounting medium (Vector Laboratories, Burlingame, CA).

Quantitation of PKC localization. For quantification of PKC translocation, random fields on the slide were selected, and the cells within each field were scored for Golgi staining (δ-PKC), perinuclear staining (δ- and ϵ-PKC), or cytoplasmic staining (ϵ-PKC). At least five fields were scored for all experiments, for a total number of at least 100 cells per slide. The observer was blind to the experimental condition of the slides.

Western blot analysis. Cells were collected in 20 mM Tris·HCl, pH 7.5, 2 mM EDTA, 10 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml each of leupeptin and aprotinin, and 0.1 mM sodium orthovanadate. Samples (1.6 mg of protein/400 μl) were each mixed with 100 μl of 5× sample buffer (25 ml of glycerol, 5.0 g of sodium dodecylsulfate, 5.2 ml of 3 M Tris, pH 6.8, 62.5 mg of Bromphenol Blue, and 12.5 ml of β-mercaptoethanol) (16) and heated for

5 min at 90°. After centrifugation at 10,000 × *g* for 10 min (4°), samples were diluted to 20–50 μg of protein and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% acrylamide gels. Proteins were transferred electrophoretically to polyvinylidene difluoride membranes that were then incubated overnight at 4° in blocking solution containing TBS (20 mM Tris·HCl, pH 7.6, 150 mM NaCl), 0.1% Tween 20, and 5% nonfat dry milk. Blots were incubated for 2 hr at room temperature with affinity-purified rabbit antibodies to PKC isozymes (0.5 mg/ml, diluted 1:200), washed three times in TBS containing 0.1% Tween 20, and then incubated with goat anti-rabbit IgG conjugated to peroxidase (1:1000). Blots were washed three times for 5 min. Immunoreactive bands were detected with an electrochemiluminscence kit (Amersham, Chicago, IL). Bands were visualized using an Epson ES-1200C Scanner (Epson America, Torrance, CA) and were quantified using the National Institutes of Health Image 1.59 PPC program.

Results

δ-PKC was localized to a Golgi-like area in approximately 70% of control NG108–15 cells (Figs. 1A and 2); in some cells, there was sparse staining for δ-PKC in the nucleus. Golgi localization of δ-PKC was confirmed by colocalization of δ-PKC with the Golgi marker BODIPY TR ceramide, exactly as described in Dohrman *et al.* (17) (data not shown). After ethanol exposure (200 mM ethanol for 48 hr), δ-PKC was localized to the perinucleus and nucleus in more than 90% of the cells and was found in the Golgi in less than 2% of the cells (Figs. 1A and 2). Ethanol-induced translocation of δ-PKC away from the Golgi to the nucleus and perinucleus was similar to that induced by activation of PKC with the phorbol ester β-PMA (100 nM for 10 min) (Fig. 1). No staining for δ-PKC was observed when the anti-δ antibody was preabsorbed with immunizing peptide before incubation with the cells (not shown).

Ethanol also induced translocation of ε-PKC in NG108–15 cells. In 95% of control cells, ε-PKC was found primarily in the perinuclear area, with low levels of staining in the nucleus (Figs. 1, 2); cytoplasmic staining or colocalization with the Golgi marker was not detected. After chronic ethanol exposure, ε-PKC was observed throughout the cytoplasm in more than 90% of the cells; perinuclear staining was still present, and nuclear staining was found in some cells (Figs. 1 and 2). In contrast to the results obtained with δ-PKC, ethanol caused translocation of ε-PKC to a site different from that in cells activated by β-PMA. β-PMA induced translocation of ε-PKC to the nucleus, not the cytoplasm (Fig. 1). Preabsorption with immunizing peptide blocked staining of cells with anti-ε-PKC antibody (not shown). The inactive phorbol ester 4α-PMA, had no effect on localization of either δ- or ε-PKC (not shown).

Our results indicate that ethanol causes translocation of both δ- and ε-PKC and that ε-PKC is translocated to a unique intracellular site distinct from that because of activation by β-PMA. The experiments with ε-PKC described below further characterize this novel finding. Maximal translocation to the cytoplasm occurred after a 48-hr incubation in 50 mM ethanol, a physiologically relevant concentration (Fig. 3A). Exposure to 25 mM ethanol for 4 days also resulted in translocation of δ-PKC to the perinuclear area and ε-PKC to the cytoplasm (Fig. 1A).

The time course for ethanol-induced translocation of ε-PKC is shown in Fig. 3B. Translocation of ε-PKC was induced by 200 mM ethanol as early as 5 min after exposure to ethanol, with maximal levels reached by 30 min (Fig. 3B). The time course for ethanol-induced translocation of δ-PKC was similar to that of ε-PKC; maximum translocation was observed at 30 min (three experiments; data not shown). ε-PKC remained in the cytoplasm as long as ethanol was present (Fig. 3B). However, 48 hr after withdrawal from ethanol, ε-PKC was again localized to the perinucleus (Fig. 1B), as in control cells. Reversible translocation of ε-PKC to the cytoplasm after brief exposure to ethanol was probably caused by translocation of existing enzyme rather than *de novo* synthesis, because Western blot analysis showed no change in ε-PKC levels at early time points. However, there was a significant increase in the amount of ε-PKC after 24 hr of exposure to 200 mM ethanol (Fig. 3C).

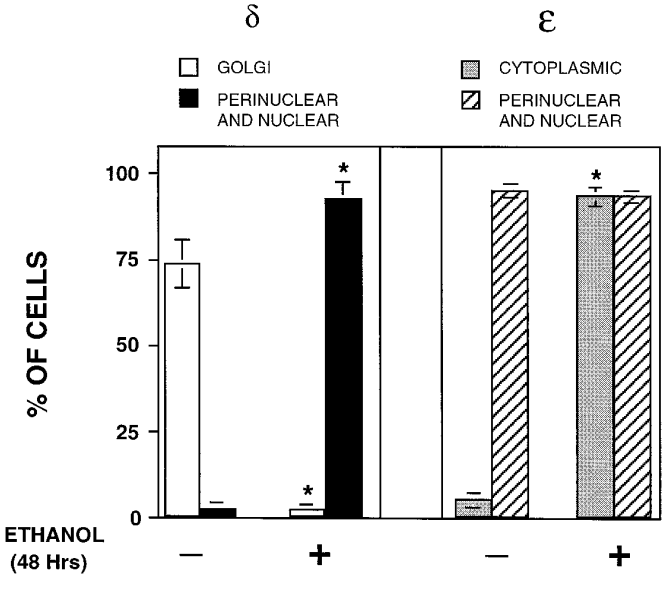

Fig. 2. Quantitation of ethanol-induced translocation of δ- and ε-PKC. NG108–15 cells were exposed to 200 mM ethanol for 48 hr and then fixed and stained for δ- and ε-PKC as described. Data shown are mean ± standard error of five experiments. *, $p < 0.05$ compared with localization in control cells (two-way ANOVA and Newman-Keul's test).

Discussion

We show here that ethanol caused translocation of δ- and ε-PKC from one intracellular site to another. PKC isozymes also translocate from one intracellular compartment to another when activated (see Ref. 14 for review). For example, δ- and ε-PKC are localized in the nucleus in unstimulated primary cardiac myocytes (18). Activation of adrenergic receptors results in translocation of δ-PKC to a filamentous network and ε-PKC to contractile elements, the perinucleus, and cell-cell contacts. Mochly-Rosen and colleagues have shown that translocation is required for activation and function of PKC isozymes (see Ref. 14 for review). Our data, therefore, suggest that ethanol-induced translocation of these isozymes reflects activation of δ- and ε-PKC. Consistent with this possibility, ethanol-induced increases in extractable PKC activity were reported in NG108–15 cells (9) and other cell types (11, 12). However, in addition to δ- and ε-PKC, other PKC isozymes are also present in these cells (8, 19); they could account for the increases in PKC activity. To determine whether δ- and ε-PKC are activated by ethanol, it will be necessary to develop antibodies to distinguish between the active and inactive states of specific PKC isozymes (20) .

Localization of PKC isozymes to specific sites and translo-

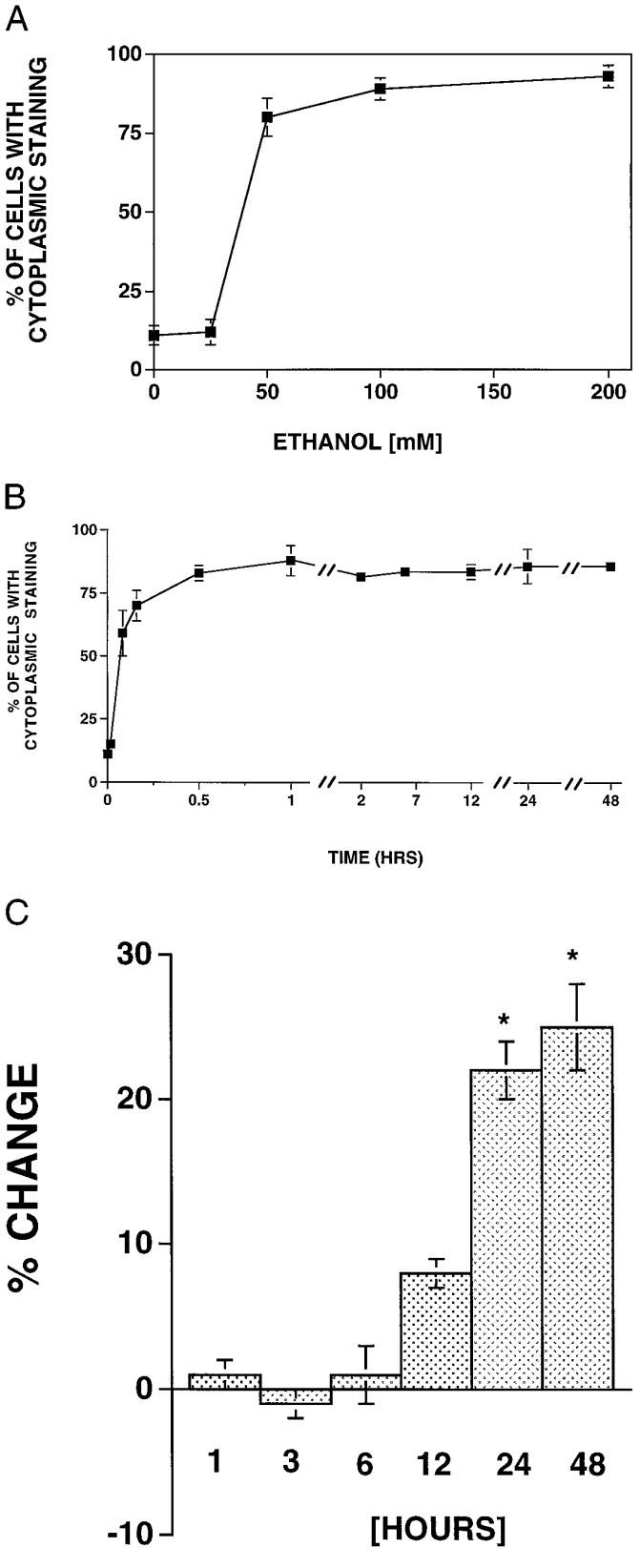

Fig. 3. Ethanol causes a time- and concentration-dependent translocation of ϵ-PKC and a time-dependent increase in ϵ-PKC amount. A, NG108–15 cells were exposed to varying concentrations of ethanol for 48 hr and then fixed and stained for ϵ-PKC as described. The percent of cells with cytoplasmic staining was determined as described (p 0.05 by ANOVA with Scheffé *post hoc* comparison; three experiments). B, NG108–15 cells were exposed to 200 mM ethanol for varying times and then fixed and stained for ϵ-PKC as described. The percent of cells with cytoplasmic staining was determined as described ($p < 0.05$ by ANOVA with Scheffé *post hoc* comparison; three experiments). C, Western blot analysis of ϵ-PKC. NG108–15 cells were exposed to 200 mM ethanol for varying times. The cells were then collected and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blots were analyzed as described. The data are presented as the percent change in the amount of ϵ-PKC in cells exposed to 200 mM ethanol relative to control cells not exposed to ethanol at varying times. Data shown are mean ± standard error; *, $p < 0.001$ (Student's t test, three experiments).

cation on activation to new sites also occurs in NG108–15 cells grown in 10% fetal calf serum (19). δ-PKC was found to be localized to neurites, the nucleus, and cytoplasm and ϵ-PKC to the cytoplasm and the perinuclear area, specifically the nuclear envelope. Stimulation by β-PMA had no effect on localization of either δ- or ϵ-PKC in these serum-grown cells. Because translocation of specific PKC isozymes by serum has been demonstrated in fibroblasts (18), it is likely that the absence of a β-PMA effect in serum-grown NG108–15 cells is attributable to prior activation and translocation of δ- and ϵ-PKC by serum. In this report, NG108–15 cells were grown in defined medium in the absence of serum. Under these conditions, δ-PKC is localized to the Golgi area, and β-PMA causes translocation to the perinuclear area and nucleus; ϵ-PKC is translocated by β-PMA from the perinuclear area to the nucleus (Figs. 1A and 2).

Ethanol caused translocation of ϵ-PKC to a different site than did β-PMA, which suggests that altered localization of ϵ-PKC may be caused by binding to an isozyme-specific anchoring receptor in the cytoplasm. Mochly-Rosen and coworkers (14, 21) identified RACKs that determine the localization and specificity of each isozyme (18, 22). Translocation of PKC isozymes to RACKs is transient (18, 22), and PKC returns to the original sites within ~60 min. This could be caused by degradation of PKC (23, 24) or receptor desensitization. In contrast, after ethanol-induced translocation, δ-and ϵ-PKC remain localized to the new intracellular sites as long as ethanol is present (Figs. 1B and 3B). It is possible, therefore, that ethanol increases the affinity of δ- and ϵ-PKC for their respective RACKs or prevents proteolytic degradation of the activated isozymes (25–27).

What is the mechanism(s) underlying ethanol-induced altered localization of δ- and ϵ-PKC? Ethanol has been reported to increase DAG levels in human epidermal keratinocytes (12). We have found a 30% increase in DAG levels after exposure of NG108–15 cells to 200 mM ethanol for 30 min.[3] This increase in DAG might be sufficient to activate and translocate δ- and ϵ-PKC because these isozymes do not require Ca^{2+} for activation. However, because ethanol causes translocation of ϵ-PKC to a site different from that caused by β-PMA activation, increases in DAG alone cannot account for the effects of ethanol on ϵ-PKC. For the same reason, it is unlikely that ethanol causes translocation of δ- and ϵ-PKC because of direct binding to the hydrophobic regulatory site on PKC (28). One possible explanation for ethanol-induced cytoplasmic localization of ϵ-PKC is that ethanol induces translocation of an ϵ-PKC-specific RACK from the perinucleus or nucleus to the cytoplasm.

Ethanol-induced translocation of δ-PKC seems to be similar to translocation induced by β-PMA. It is likely, then, that ethanol causes activation of δ-PKC and that "normal" substrates

[3] Wu, Z.-L, unpublished observations.

are phosphorylated. Translocation of δ-PKC to the nucleus might contribute to ethanol-induced changes in gene transcription, as reported in NG108–15 cells (29–31) and in rat brain (32–34). Unlike δ-PKC, however, the localization of ϵ-PKC is dramatically different in ethanol-treated cells compared with β-PMA-treated cells. Ethanol causes ϵ-PKC translocation to the cytoplasm, whereas β-PMA causes ϵ-PKC translocation to the nucleus. If ϵ-PKC were activated by ethanol, it would be expected to phosphorylate and thereby regulate the function of cytoplasmic proteins not normally regulated by this isozyme and thereby alter cellular functions.

As discussed above, our data suggest that δ- and ϵ-PKC are activated by ethanol. However, even if δ- and ϵ-PKC are not active at the new sites in ethanol-treated cells, there may be altered responses to physiologic signals that ordinarily activate these isozymes. For example, when ethanol-treated cells are activated by neurotransmitters or other signaling molecules, δ-and ϵ-PKC would be expected to phosphorylate substrates in the nucleus and cytoplasm rather than at the Golgi or perinucleus, respectively.

We have reported recently that ethanol causes translocation of PKA from the Golgi area to the nucleus (17) in NG108–15 cells. Ethanol-induced translocation of PKA to the nucleus should also have profound effects on cellular signaling and gene expression and on other cellular pathways regulated by PKA. Moreover, we have shown that the loss of ethanol sensitivity of adenosine transport after chronic exposure to ethanol is mediated by alterations in both PKA (35) and PKC (8) that may be caused by cross-talk between these two pathways. Taken together, our results suggest that ethanol alters the localization of several key protein kinases and that this altered localization could account for many of the pleiotropic effects of ethanol on cellular functions.

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