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**Dopamine and Ethanol Cause Translocation of  $\epsilon$ PKC Associated with  $\epsilon$ RACK:**

**Cross-talk Between PKA and PKC Signaling Pathways**

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**Abbreviations:** PKC, protein kinase C; PKA, protein kinase A; RACK, receptor for activated C-kinase; DAG, diacylglycerol; D2, dopamine D2 receptor; A2A, adenosine A2A receptor; PLC, phospholipase C; PGE1, prostaglandin E1; IgG, immunoglobulin G; NPA, 2, 10, 11-trihydroxy-*N*-propylnorapomorphine hydrobromide; BSA, bovine serum albumin.

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## ABSTRACT

Previously we found that neural responses to ethanol and the dopamine D2 receptor (D2) agonist NPA involve both epsilon protein kinase C ( $\epsilon$ PKC) and cAMP-dependent protein kinase A (PKA). However, little is known about the mechanism underlying ethanol- and D2-mediated activation of  $\epsilon$ PKC and the relationship to PKA activation. In the present study, we used a new  $\epsilon$ PKC antibody, 14E6, that selectively recognizes active  $\epsilon$ PKC when not bound to its anchoring protein  $\epsilon$ RACK (receptor for activated C-kinase), and PKC isozyme-selective inhibitors and activators, to measure PKC translocation and catalytic activity. We show here that ethanol and NPA activated  $\epsilon$ PKC and also induced translocation of both  $\epsilon$ PKC and its anchoring protein,  $\epsilon$ RACK to a new cytosolic site. The selective  $\epsilon$ PKC agonist, pseudo- $\epsilon$ RACK, activated  $\epsilon$ PKC but did not cause translocation of the  $\epsilon$ PKC/ $\epsilon$ RACK complex to the cytosol. These data suggest a step-wise activation and translocation of  $\epsilon$ PKC following NPA or ethanol treatment where  $\epsilon$ PKC first translocates and binds to its RACK and subsequently the  $\epsilon$ PKC/ $\epsilon$ RACK complex translocates to a new subcellular site. Direct activation of PKA by Sp-cAMPS, PGE1 or the adenosine A2A receptor is sufficient to cause  $\epsilon$ PKC translocation to the cytosolic compartment in a process that is dependent on PLC activation and requires PKA activity. These data demonstrate a novel cross-talk mechanism between  $\epsilon$ PKC and PKA signaling systems. PKA and PKC signaling have been implicated in alcohol rewarding properties in the mesolimbic dopamine system. Cross-talk between PKA and PKC may underlie some of the behaviors associated with alcoholism.

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## INTRODUCTION

Intracellular signal transduction cascades linked to protein kinase C (PKC) have been implicated in drug abuse (Choi et al., 2002; Hodge et al., 1999; Newton and Messing, 2006; Olive et al., 2000; Olive and Messing, 2004). In particular the isozyme  $\epsilon$ PKC mediates an intracellular response to ethanol (Gordon et al., 2001; Gordon et al., 1997) and is associated with excessive drinking.  $\epsilon$ PKC knockout mice exhibit decreased alcohol consumption in two bottle-choice and operant self-administration paradigms (Hodge et al., 1999; Olive et al., 2000). In addition, conditional expression of  $\epsilon$ PKC in the basal forebrain, amygdala, and cerebellum of  $\epsilon$ PKC knockout mice restored the wild-type response to alcohol (Choi et al., 2002).

Stimulation of cells with hormones or neurotransmitters that trigger diacylglycerol (DAG) formation causes activation and translocation of PKC from one subcellular site to another (Mochly-Rosen and Gordon, 1998). Translocation of PKC is associated with anchoring of the activated enzyme to selective receptors for activated C-kinase (RACKs); the functional selectivity of each activated PKC isozyme is determined by its binding to a corresponding RACK (Mochly-Rosen and Gordon, 1998). However, it is not clear how the active enzyme translocates to its functional site where its RACK is located and what other enzymes may be involved in the activation and translocation process.

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Alcohol and other addictive drugs appear to converge on specific dopaminergic pathways in the mid-brain. In particular, dopamine D2 receptors (D2) have been implicated in the rewarding properties of these drugs (Robbins and Everitt, 1999; Volkow et al., 2004). We previously demonstrated in NG108-15/D2 cells that ethanol and the D2 agonist NPA cause translocation of  $\epsilon$ PKC from the perinuclear region to the cytoplasm (Gordon et al., 2001; Gordon et al., 1997).  $\epsilon$ PKC translocation in ethanol-stimulated cells reached maximum at 30 min, while NPA-induced  $\epsilon$ PKC translocation was maximal at 10 min (Gordon et al., 2001; Gordon et al., 1997). In these cells, ethanol and NPA also activated cAMP-dependent protein kinase A (PKA) (Dohrman et al., 2002; Yao et al., 2002); this activation also occurred within the first minute of stimulation (Dohrman et al., 2002; Yao et al., 2002). PKA is localized at the Golgi apparatus (Dohrman et al., 1996), near the location of  $\epsilon$ PKC in unstimulated cells (Gordon et al., 2001; Gordon et al., 1997). In this current study, we found that  $\epsilon$ PKC binding to  $\epsilon$ RACK precedes its translocation and that PKA is required for the translocation of the  $\epsilon$ PKC/ $\epsilon$ RACK complex.

### MATERIALS AND METHODS

**Materials.** All reagents were purchased from Sigma (St. Louis, MO) except where indicated. Rp-cAMPS and Sp-cAMPS were purchased from BioLog (La Jolla, CA). Bisindolylmaleimide I (GF109203X) and Et-18-OCH<sub>3</sub> were purchased from Calbiochem (San Diego, CA). 2, 10, 11-trihydroxy-*N*-propylnorapomorphine hydrobromide (NPA) was purchased from Research Biochemicals Inc. Protease inhibitor tablets (complete) were purchased from Roche Molecular Biochemicals (Indianapolis, IN).

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**Cell Culture.** NG108-15 cells stably expressing the rat D2L receptor (NG108-15/D2) (Asai et al., 1998) were grown on single-well slides in defined media for 2 days followed by daily replacement until day 4 (Dohrman et al., 1996). The cells were treated as described in the figure legends and fixed as described below (Gordon et al., 1997).

**Immunocytochemistry and Microscopy.** Cells were fixed with cold methanol for 2 to 3 min and rinsed 3 times with PBS, incubated at room temperature with blocking buffer (1% normal goat serum in PBS and 0.1% Triton X-100) for 3 to 4 h, and then incubated overnight at 4°C in PBS containing 0.1% Triton X-100, 2 mg/ml fatty acid-free bovine serum albumin (Dohrman et al., 1996), primary antibodies specific for  $\epsilon$ PKC (mouse IgG raised against the V5 domain of  $\epsilon$ PKC, Santa Cruz Biotechnology, Santa Cruz, CA),  $\epsilon$ RACK (rat IgG, Stressgen, Victoria, BC, Canada) for  $\epsilon$ RACK, and 14E6 (mouse IgM, raised against the V1 domain of  $\epsilon$ PKC) for active  $\epsilon$ PKC (Souroujon et al., 2004). The cells were then washed three times with PBS, incubated for 1 h at room temperature with goat anti-mouse IgM, anti-mouse IgG, or anti-rat IgG secondary antibodies (Cappel, Aurora, OH) (diluted 1:1000), washed three times with PBS, and coverslipped with Vectashield mounting medium. Cells were imaged using a Bio-Rad 1024 scanning laser confocal microscope equipped with a krypton-argon laser attached to a Nikon Optiphot microscope. Images were collected as z-series using Kalman averaging of scans (Gordon et al., 1997). Collected data were processed using NIH Image and Adobe Photoshop software (Adobe, Mountain View, CA). All images were obtained under 40x magnification from individual middle sections of the projected z-series.

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**Quantification of PKC Localization.** Fields on each slide were selected at random and cells scored for perinuclear or cytoplasmic staining by two independent observers who were blind to the experimental conditions. At least four fields were scored for each experiment, for a total number of at least 50 cells per slide.

**Cell Fractionation.** NG108-15/D2 cells in 100 mm dishes ( $2 \times 10^6$  cells/dish) were incubated with ethanol or NPA for 10 min, washed with cold PBS and lysed on ice in 0.5 ml lysis buffer containing 50 mM Tris-HCL (pH 7.4), 2.5 mM MgCL<sub>2</sub>, 1 mM EDTA, 1 mM DTT, 10% glycerol and protease inhibitors (0.1 mM phenylmethyl sulfonyl fluoride, 20 µg/ml soybean trypsin inhibitor, 25 µg/ml aprotinin, 25 µg/ml leupeptin, and 1 mM sodium orthovanadate). Cells were homogenized by ten passes through a 26-gauge needle and centrifuged at 3000 rpm for 5 min at 4°C. The supernatant was centrifuged for 20 min at  $150,000 \times g$  to separate the membrane pellet from the cytosol (Yao et al., 2002). The supernatant was saved as the cytosolic fraction. The remaining pellets were suspended in 0.5 ml of lysis buffer containing 0.1% Triton-X 100, titrated and incubated on ice for 20 min. This suspension was centrifuged as described above, and the Triton-soluble material was collected as the original particulate fraction.

**Immunoprecipitation and Western Blot.** 5 µg of εPKC monoclonal IgG antibody was incubated with 50 µl of protein A/G beads (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C. Antibody-bound beads were then washed twice with PBS and blocked with 3% BSA for 2 h at 4 °C. The cytosolic fraction was precleared with protein A/G beads for 30 min at 4 °C, incubated with the antibody-bound beads overnight at 4 °C and

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subsequently washed four times with PBS. Bound material was eluted with SDS sample buffer, run on a 10% SDS/PAGE and transferred and probed for  $\epsilon$ PKC (mouse IgG, Santa Cruz, CA) and  $\epsilon$ RACK (rat IgG, Victoria, BC). Secondary antibody was horseradish peroxidase-linked goat anti-mouse or anti-rat (1:1000) (NEN BioLabs, Beverly, MA). Proteins were detected using LumiGLO chemiluminescence substrate (NEN BioLabs).

**PKC Activity Assay.** Cells grown in 100 mm plates were treated with ethanol or NPA for 10 min, washed with cold PBS, harvested in 1 ml whole cell lysis buffer (20 mM Tris, pH 7.5, 2 mM EDTA, 10 mM EGTA, 0.1% Triton-X 100, and 1 tablet of protease inhibitor/10 ml), and lysed on ice for 20 min. The lysate was centrifuged at 14,000 rpm for 10 min in an Eppendorf centrifuge. The supernatant was immunoprecipitated for  $\epsilon$ PKC as described above. To assay  $\epsilon$ PKC activity, immunoprecipitates were incubated at 30 °C for 20 min with 10  $\mu$ M ATP, 0.5  $\mu$ ci [ $\gamma$ - $^{32}$ P]ATP and a peptide substrate mixture from SignATECT PKC Assay System (Promega, Madison, WI). PKC activity was detected as described by the manufacturer.

## RESULTS

### ***Ethanol and NPA both cause translocation of $\epsilon$ PKC and $\epsilon$ RACK to the same location.***

Ethanol and the D2 agonist NPA cause translocation of  $\epsilon$ PKC (Gordon et al., 2001; Gordon et al., 1997). Activated  $\epsilon$ PKC associates with the  $\epsilon$ RACK,  $\beta'$ -COP (Csukai et al., 1997). To determine whether  $\epsilon$ RACK translocates together with  $\epsilon$ PKC, NG108-15/D2 cells were treated with either ethanol or the D2 agonist NPA for 10 min and analyzed for

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translocation of  $\epsilon$ PKC and  $\epsilon$ RACK. Fig. 1A shows that ethanol and NPA both induced  $\epsilon$ PKC (green) translocation from the nucleus/perinucleus to the cytoplasm, and  $\epsilon$ RACK (red) from the Golgi/perinucleus to the cytoplasm. The merged images (yellow, Fig. 1A) indicate that  $\epsilon$ PKC and  $\epsilon$ RACK are co-localized in the cytoplasm in ethanol- and NPA-treated cells. Co-translocation and association of the two proteins was confirmed by co-immunoprecipitation. Western blot analysis showed that the amount of  $\epsilon$ PKC in the cytosolic compartment increased concomitantly with the amount of  $\epsilon$ RACK (Fig. 1B), suggesting that  $\epsilon$ PKC and  $\epsilon$ RACK moved together after treatment with either ethanol or NPA.

### ***PKA activation is required and sufficient to cause $\epsilon$ PKC and $\epsilon$ RACK translocation.***

Since ethanol and NPA also activate PKA, and PKA translocation is more rapid than  $\epsilon$ PKC (Dohrman et al., 1996; Gordon et al., 1998; Gordon et al., 2001; Yao et al., 2002), we asked whether PKA is required for  $\epsilon$ PKC and  $\epsilon$ RACK translocation and co-localization. Fig. 1A and 1B show that the PKA inhibitor, Rp-cAMPS, prevents the translocation of both  $\epsilon$ PKC and  $\epsilon$ RACK as the distribution of  $\epsilon$ PKC and  $\epsilon$ RACK appears the same as in control cells. In contrast, NPA- and ethanol-induced translocation of  $\delta$ PKC was not affected by Rp-cAMPS (data not shown). To investigate how PKA regulates ethanol- and NPA-induced  $\epsilon$ PKC and  $\epsilon$ RACK translocation, we determined whether activation of PKA is sufficient for  $\epsilon$ PKC and  $\epsilon$ RACK translocation. Fig. 2 shows that the PKA activator, Sp-cAMPS, or activation of the  $G\alpha_s$ -coupled PGE1 receptor each causes

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translocation of  $\epsilon$ PKC and  $\epsilon$ RACK to the cytoplasm, similar to ethanol and NPA treatments (Fig. 1A). We previously demonstrated that ethanol activates PKA via adenosine A2A receptors (A2A) (Yao et al., 2002). To determine whether direct activation of the adenosine A2A receptor causes translocation of  $\epsilon$ PKC/ $\epsilon$ RACK, cells were treated with an adenosine A2A agonist CGS21680 for 10 min. We found that CGS21680 mimics ethanol-induced translocation of  $\epsilon$ PKC/ $\epsilon$ RACK (Fig. 1C). This translocation was blocked by the A2A antagonist DMPX or the PKA inhibitor Rp-cAMPS (Fig. 1C). Rp-cAMPS and DMPX had no effect on the localization of  $\epsilon$ PKC and  $\epsilon$ RACK in unstimulated cells (data not shown).

Since PKA directly activates PLC, increases DAG levels, and results in activation and translocation of PKC in LTK/D1 cells (Yu et al., 1996), we asked whether PKA activates  $\epsilon$ PKC *via* PLC in NG108-15/D2 cells. We found that the PLC inhibitor, Et-18-OCH<sub>3</sub> without effect in unstimulated cells (data not shown), inhibits Sp-cAMPS- or PGE<sub>1</sub>-induced translocation of  $\epsilon$ PKC and  $\epsilon$ RACK (Fig. 2). As expected, Rp-cAMPS also prevented these translocations (Fig. 2).

***Ethanol and NPA cause translocation of  $\epsilon$ PKC/ $\epsilon$ RACK complex via the PLC/PKC system.*** We have previously shown that ethanol- or NPA-induced translocation of  $\epsilon$ PKC is blocked by the PLC inhibitor Et-18-OCH<sub>3</sub> (Gordon et al., 2001). If PLC activation is required for ethanol- and NPA-induced translocation of  $\epsilon$ PKC, then inhibition of PLC activity should also inhibit translocation of  $\epsilon$ PKC/ $\epsilon$ RACK. Indeed, the PLC inhibitor Et-

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18-OCH<sub>3</sub> blocked  $\epsilon$ RACK translocation along with  $\epsilon$ PKC (Fig. 2 and 3). As anticipated, the PKC inhibitor GF 109203X also blocked translocation (data not shown).

Because both  $G\alpha$  and  $\beta\gamma$  released from trimeric G proteins can stimulate PLC $\beta$  isozymes (Camps et al., 1992; Park et al., 1993; Runnels and Scarlata, 1999) and PTX inhibits ethanol- and NPA-induced translocation of  $\epsilon$ PKC (Gordon et al., 2001), we next asked whether  $\epsilon$ RACK translocation requires  $G\alpha_i$ . We found that PTX, which inhibits  $G\alpha_i/o$  and  $\beta\gamma$ , prevented co-translocation of  $\epsilon$ RACK with  $\epsilon$ PKC (Fig. 3). We know that the A<sub>2A</sub> antagonist DMPX blocks ethanol- but not NPA-induced  $\epsilon$ PKC translocation and that the D<sub>2</sub> antagonist spiperone blocks NPA- but not ethanol-induced  $\epsilon$ PKC translocation (Gordon et al., 2001). Here, we show that DMPX or spiperone each prevents ethanol- or NPA-induced  $\epsilon$ RACK translocation separately (Fig. 3). In contrast, PTX, DMPX or spiperone alone was without effect on the localization of  $\epsilon$ PKC and  $\epsilon$ RACK in unstimulated cells (data not shown). Taken together, these findings suggest that ethanol, *via* the adenosine A<sub>2A</sub> receptor, and dopamine, *via* the D<sub>2</sub> receptor, cause  $\epsilon$ PKC and  $\epsilon$ RACK translocation by stimulating the PLC/PKC signaling pathway.

***$\epsilon$ PKC activation is required for translocation of  $\epsilon$ PKC and  $\epsilon$ RACK.*** To further investigate whether  $\epsilon$ PKC activation regulates the translocation of  $\epsilon$ PKC and  $\epsilon$ RACK, we utilized an IgM monoclonal antibody, 14E6, that specifically detects the active conformation of  $\epsilon$ PKC (Souroujon et al., 2004). Fig. 4A,B show that translocation of  $\epsilon$ PKC (green) together with  $\epsilon$ RACK (blue) began at 1 min, and persists for 30 min after

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the addition of ethanol and NPA. In contrast,  $\epsilon$ PKC staining with 14E6 (red) increased within 1 min, maximized by 10 min, and returned to the basal level by 30 min (Fig. 4A,B).  $\epsilon$ PKC translocation was observed at the time when 14E6 staining appeared. These data suggest that  $\epsilon$ PKC activation appears to be required for the translocation of  $\epsilon$ PKC and  $\epsilon$ RACK. Consistent with our published observations (Souroujon et al., 2004),  $\epsilon$ PKC activation precedes its binding to  $\epsilon$ RACK and its translocation with  $\epsilon$ RACK to the cytoplasm. Translocation of  $\epsilon$ PKC persisted at 30 min when the activated enzyme was no longer detected by 14E6 (Fig. 4A,B), suggesting that the 14E6 epitope (V1 domain, the RACK-binding domain) becomes inaccessible when  $\epsilon$ PKC is bound to  $\epsilon$ RACK (Souroujon et al., 2004). We confirmed these findings by directly measuring the catalytic activity of  $\epsilon$ PKC. In accordance with translocation,  $\epsilon$ PKC activity peaked at 10 min, persisted at 30 min and returned to the basal level at 60 min (Fig. 4C).

To determine which PKC isozymes mediate ethanol- or NPA-induced translocation of  $\epsilon$ PKC and  $\epsilon$ RACK, cells were treated with isozyme-selective translocation peptide inhibitors:  $\epsilon$ V1-2 for  $\epsilon$ PKC,  $\delta$ V1-1 for  $\delta$ PKC or  $\beta$ C2-4 for conventional PKC (Schechtman and Mochly-Rosen, 2002) respectively, prior to the treatment of ethanol or NPA. We found that  $\epsilon$ V1-2, but not  $\delta$ V1-1 or  $\beta$ C2-4, prevented ethanol- or NPA-induced translocation of  $\epsilon$ PKC and  $\epsilon$ RACK (Fig. 5A,B). In control experiments, these peptide inhibitors did not alter the localization of  $\epsilon$ PKC and  $\epsilon$ RACK in unstimulated cells (data not shown). These results suggest that  $\epsilon$ PKC activation is solely responsible for ethanol- and NPA-induced translocation of  $\epsilon$ PKC and  $\epsilon$ RACK.

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***εPKC activation and translocation with εRACK are separate events.*** It appears that ethanol and NPA induce translocation of εPKC and εRACK *via* the PLC/PKC system. However, we found that these translocations are also PKA-dependent. To understand the role of PKC and PKA in this process, cells were treated with an εPKC agonist pseudo-εRACK (ψεRACK) that activates only εPKC (Schechtman and Mochly-Rosen, 2002). A 10 min incubation with pseudo-εRACK activated εPKC, as detected by 14E6 (red). Some εPKC translocates from the nucleus to the perinucleus, where it appears to bind to εRACK (pink). However, activated εPKC did not translocate further to the cytoplasmic compartment (Fig. 6A). Cells treated with NPA, which stimulates both PKA and PKC, showed translocation of εPKC and εRACK to this cytoplasmic compartment (Fig. 6A). Moreover, the PKA inhibitor, Rp-cAMPS, blocked NPA-induced translocation of εPKC and εRACK, but did not affect activation of εPKC as measured by 14E6 staining (Fig. 6A). Western blot analysis confirmed that NPA, but not pseudo-εRACK, caused εPKC translocation from the particulate to the cytosolic fraction (Fig. 6B). These results suggest that the complex εRACK/εPKC does not translocate further into the cytosolic compartment unless PKA is active. It appears that εPKC activation and anchoring to its RACK and translocation of the εPKC/εRACK complex are separate events.

## DISCUSSION

The major findings in this study are that ethanol and NPA each can induce translocation of εPKC and εRACK to a new site and that this process requires PKA

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activity. Following stimulation,  $\epsilon$ PKC translocates from the perinucleus/nucleus to a new perinuclear/Golgi compartment, perhaps where  $\epsilon$ RACK is colocalized in unstimulated cells. Subsequently,  $\epsilon$ PKC and the  $\epsilon$ RACK translocate from the perinucleus/Golgi to the cytosol. Translocation of  $\epsilon$ PKC and  $\epsilon$ RACK to the cytosol occurs only when PKA is activated, a process that is  $G\alpha_i$ -dependent. Consistent with this observation, the  $\epsilon$ PKC agonist, pseudo- $\epsilon$ RACK, did not cause the translocation of  $\epsilon$ PKC to the cytosol although it activated  $\epsilon$ PKC. Moreover, activation of PKA by Sp-cAMPS, PGE1 or the adenosine A2A receptor alone is sufficient to cause  $\epsilon$ PKC and  $\epsilon$ RACK translocation. Importantly, PKA-dependent translocation of  $\epsilon$ PKC was inhibited by the PLC inhibitor Et-18-OCH<sub>3</sub>, suggesting that in addition to the PLC-mediated cross-talk between PKC and PKA signaling, there is a second cross-talk event leading to translocation of  $\epsilon$ PKC/ $\epsilon$ RACK complex that is dependent on PKA activity. Therefore, there is a dual requirement for PKA activity in PKC signaling. A schematic model for ethanol and D2 activation of PKA/PKC cross-talk is presented in Fig. 7.

**PKA.** Our findings in this study complement our earlier observations that incubation with ethanol or NPA causes the catalytic subunit (C $\alpha$ ) of PKA to translocate from the Golgi to the cytoplasm and nucleus (Dohrman et al., 1996; Yao et al., 2002; Yao et al., 2003). We have shown that acute ethanol-induced PKA C $\alpha$  translocation appears to be due to an ethanol-induced increase of extracellular adenosine, which activates adenosine A2A receptors to promote cAMP production (Yao et al., 2002) (Fig. 7). We

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have also shown that NPA-induced PKA C $\alpha$  translocation is likely due to  $\beta\gamma$  activation of adenylyl cyclase (AC) II and/or IV, because PTX and  $\beta\gamma$  scavenger peptide prevent PKA C $\alpha$  translocation (Yao et al., 2002; Yao et al., 2003).  $\beta\gamma$  activation of AC II or IV requires either G $\alpha$ s (Baker et al., 1999; Federman et al., 1992) or PKC (Tsu and Wong, 1996). This is consistent with our observations that A2A and D2 agonists each activate cAMP production via G $\alpha$ s and  $\beta\gamma$ , respectively. Moreover, the PKC inhibitor bisindolylmaleimide I (GF 109203X) blocks PKA C $\alpha$  translocation induced by NPA, but not by ethanol (Yao et al., unpublished observation).

**PKC and RACK.** Ethanol and NPA also induce translocation of  $\epsilon$ PKC. 100 mM ethanol induced maximal translocation at 10 min without affecting cell morphology and viability. 50 mM ethanol produced maximal translocation at 48 hrs (Gordon et al., 2001; Gordon et al., 1997). Therefore, we chose 100 mM ethanol and a 10 min incubation time as optimal conditions to define the mechanism and relationship between  $\epsilon$ PKC activation and translocation. Using the antibody 14E6, we show that ethanol and NPA activate  $\epsilon$ PKC and increase the catalytic activity of  $\epsilon$ PKC measured by phosphorylation. We also show that  $\epsilon$ V1-2, an inhibitor of  $\epsilon$ PKC binding to its RACK, prevents ethanol- and NPA-induced translocation of the  $\epsilon$ PKC/ $\epsilon$ RACK complex. In contrast, the peptide inhibitor  $\delta$ V1-1 ( $\delta$ PKC) or  $\beta$ C2-4 (classical PKC) had no effect. We previously proposed that the site of localization of activated PKC isozymes is determined by the location of isozyme-specific RACKs (Mochly-Rosen and Gordon, 1998). Our data suggest that ethanol and

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NPA utilize this mechanism to relocate activated  $\epsilon$ PKC. The activated  $\epsilon$ PKC binds first to its RACK and subsequently translocates from the perinucleus to a new cytoplasmic compartment. Thus, activation of  $\epsilon$ PKC appears to be necessary for  $\epsilon$ PKC and  $\epsilon$ RACK translocation (Fig. 7). However, activation of  $\epsilon$ PKC alone is not sufficient to cause translocation of the  $\epsilon$ PKC/ $\epsilon$ RACK complex because the  $\epsilon$ PKC agonist, pseudo- $\epsilon$ RACK, does not cause translocation of  $\epsilon$ PKC into the cytoplasm despite activating  $\epsilon$ PKC. These observations demonstrate that PKA activation induced by ethanol or NPA has a dual role in  $\epsilon$ PKC signaling: first PKA activates PLC to produce DAG for  $\epsilon$ PKC activation and second, PKA causes relocation of activated  $\epsilon$ PKC/ $\epsilon$ RACK. This is likely to yield different cellular responses, as the protein substrates of  $\epsilon$ PKC should be different in each of these cellular locations.

**Cross-talk between PKA and PKC signaling.** Cross-talk between PKA and PKC signaling pathways is increasingly recognized as a mechanism to regulate signal transduction cascades. However, the molecular events underlying PKA/PKC cross-talk are not clear. Recent work suggests a role for PKA in the activation and translocation of PKC (Huang et al., 2001; Yu et al., 1996). PKA-dependent activation of PKC also occurs in B lymphocytes (Cambier et al., 1987). In addition, activation of dopamine D1 receptors, known to couple to  $G\alpha_s$ , increases PKC activity and translocation in LTK cells (Yu et al., 1996). In this study, we demonstrate that **translocation** of  $\epsilon$ PKC and  $\epsilon$ RACK by ethanol and NPA requires PKA activation but the PKA inhibitor Rp-cAMPS does not

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inhibit the **activation** of  $\epsilon$ PKC. These findings suggest that PKA may regulate the location of  $\epsilon$ PKC/ $\epsilon$ RACK complex while not affecting the activation state of the enzyme (Fig. 7). Indeed, prosite analysis reveals a consensus PKA phosphorylation site in  $\epsilon$ RACK (our unpublished observation). Thus, not only do RACKs bind activated PKC isozymes, but RACK phosphorylation may further regulate its translocation to intracellular sites.

One of our most surprising findings is that robust activation of PKA by Sp-cAMPS or PGE1 was sufficient to induce translocation of  $\epsilon$ PKC and  $\epsilon$ RACK. Importantly, direct activation of the adenosine A2A receptor by CGS21680 also caused translocation of  $\epsilon$ PKC/ $\epsilon$ RACK to the same compartment. We propose that activation of PKA stimulates PLC $\beta$ , thus increasing DAG levels and causing activation and translocation of  $\epsilon$ PKC (Fig. 7). Indeed, a PLC inhibitor blocks  $\epsilon$ PKC translocation. However, it is still unclear how ethanol, NPA or PKA activate the correct pool of PKC and how activated  $\epsilon$ PKC translocates with  $\epsilon$ RACK to its functional intracellular sites. One explanation is a “targeting hypothesis”, that phosphorylation events are controlled in part by the intracellular location of specific kinases in the cell (Hubbard and Cohen, 1993). It has also been suggested that intracellular anchoring proteins regulate cell signaling dynamics in time and space. The Golgi complex is a major subcellular location for PKA in mammalian cells (Dohrman et al., 1996; Nigg et al., 1985; Yao et al., 2002) and is involved in vesicle-mediated protein transport processes (Muniz et al., 1997). Scott and collaborators and others suggest that some anchoring proteins for PKA, collectively termed AKAPs for A Kinase Anchoring Proteins, also bind inactive PKC in

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the Golgi (Faux and Scott, 1997; Pawson and Scott, 1997). Also,  $\epsilon$ RACK,  $\beta'$ -COP, is a coatomer protein which moves with vesicles and localizes at the Golgi apparatus (Salama and Schekman, 1995). Thus, AKAPs such as AKAP 350 may act as a scaffold protein that binds PKA,  $\epsilon$ PKC and  $\epsilon$ RACK (Diviani and Scott, 2001; Shanks et al., 2002) in the Golgi and serves as a platform to organize and regulate PKA and PKC interactions. It remains to be determined which AKAP binds to PKA,  $\epsilon$ PKC, and  $\epsilon$ RACK, and how AKAP targets PKA and  $\epsilon$ PKC to discrete intracellular locations and coordinates multiple components of signal transduction pathways.

**Relevance to alcoholism.** Our results provide new insight into some of the cellular events mediated by ethanol and dopamine. Ethanol causes the release of dopamine in the brain (Imperato and Di Chiara, 1986; McBride et al., 1993) and presumably dopamine acts on D2 to mediate rewarding properties of ethanol. We show here that both ethanol and a D2 agonist activate both PKA and PKC signaling pathways *via* a complex cross-talk between these two signaling cascades. It is tempting to speculate that ethanol and D2 may activate the same signaling pathways since they synergistically activate PKA and PKC signaling (Gordon et al., 2001; Yao et al., 2002). Moreover, ethanol- and dopamine-regulated translocation of PKA and  $\epsilon$ PKC appears to play a role in drinking behaviors; mice lacking  $\epsilon$ PKC show reduced operant ethanol self-administration (Hodge et al., 1999; Olive et al., 2000) and inhibition of the cAMP/PKA signaling pathway generally increases sensitivity to ethanol sedation and reduces ethanol preference and consumption (Moore et al., 1998; Wand et al., 2001; Yao et al., 2002).

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Taken together with the results in this study, it is possible that drugs which interfere with PKA and PKC cross-talk might be potential therapeutics for alcoholism.

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### FOOTNOTES

Daria Mochly-Rosen is a founder of KAI Pharmaceuticals, a company that plans to bring PKC regulators to the clinic. However, this work was carried out in her university laboratory, with the sole support of NIH AA11147. This research was also supported by NIH AA010030-12 to I.D. and L.Y.

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## FIGURE LEGENDS

**Fig. 1. Ethanol- and NPA-induced translocation of  $\epsilon$ PKC and  $\epsilon$ RACK requires PKA activity in NG108-15/D2 cells.** *A*, NG108/15 cells expressing the D2 were exposed to 100 mM ethanol (EtOH) or the D2 agonist, NPA (50 nM) for 10 min. Where indicated, cells were preincubated with or without the PKA inhibitor, Rp-cAMPS (Rp, 20  $\mu$ M) for 20 min before the treatment of ethanol or NPA. Cells were fixed and stained for  $\epsilon$ PKC (green, mouse IgG against the V5 domain of  $\epsilon$ PKC, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and  $\epsilon$ RACK (red), and scanned using a Bio-Rad 1024 confocal microscope. The merged images of  $\epsilon$ PKC with  $\epsilon$ RACK suggest the co-localization (yellow). The data are representative of at least three independent experiments. Preabsorption of the isozyme-specific antibody with the respective peptide antigens were carried out as described in Gordon et al (Gordon et al., 1997). *B*, Cytosolic fractions were prepared from cells treated with 100 mM ethanol or 50 nM NPA for 10 min, immunoprecipitated, and probed with the mouse  $\epsilon$ PKC or a monoclonal  $\epsilon$ RACK antibody. *C*, Cells were preincubated with or without the PKA inhibitor, Rp-cAMPS (Rp, 20  $\mu$ M) or the adenosine A2A antagonist, DMPX (10  $\mu$ M) for 20 min and then treated with the A2A agonist, CGS21680 (CGS, 100 nM) for 10 min. Immunoprecipitation and detection of  $\epsilon$ PKC/ $\epsilon$ RACK translocation were performed as *B*.

**Fig. 2. PKA stimulates PLC to induce  $\epsilon$ PKC and  $\epsilon$ RACK translocation.** NG108-15/D2 cells were preincubated with or without the PLC inhibitor, Et-18-OCH3 (Et-18, 10

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$\mu\text{M}$ ) or the PKA inhibitor, Rp-cAMPS (Rp, 20  $\mu\text{M}$ ) for 20 min and then treated with 1 mM Sp-cAMPS (Sp) or 10  $\mu\text{M}$  PGE1 for 10 min. Analyses of  $\epsilon\text{PKC}$  and  $\epsilon\text{RACK}$  translocation were performed as Fig. 1A.

### **Fig. 3. Ethanol- and NPA- induced $\epsilon\text{PKC}$ and $\epsilon\text{RACK}$ translocation requires PLC.**

NG108–15/D2 cells were preincubated with or without the PLC inhibitor Et-18 (10  $\mu\text{M}$ ), the A2A antagonist DMPX (10  $\mu\text{M}$ ), or the D2 antagonist spiperone (SPIP, 10  $\mu\text{M}$ ) for 30 min, or PTX (50 ng/ml) overnight, and then treated with 100 mM ethanol or 50 nM NPA for 10 min. Immunostaining for  $\epsilon\text{PKC}$  and  $\epsilon\text{RACK}$  translocation were carried out as Fig. 1A.

### **Fig. 4. $\epsilon\text{PKC}$ and $\epsilon\text{RACK}$ translocation as a function of time.**

NG108-15/D2 cells were incubated with 100 mM ethanol (A) or 50 nM NPA (B) for the indicated times and stained for  $\epsilon\text{PKC}$  (green), activated  $\epsilon\text{PKC}$  (14E6, red) and  $\epsilon\text{RACK}$  (blue). The merged images were produced using anti-mouse IgG against the V5 domain of  $\epsilon\text{PKC}$  (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), state-specific anti- $\epsilon\text{PKC}$  antibodies, 14E6, and anti- $\epsilon\text{RACK}$  antibodies. C, Cells were lysed and immunoprecipitated with anti-mouse  $\epsilon\text{PKC}$  antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The immunoprecipitates were assayed for PKC phosphorylation activity as described in material and methods. Data are the mean  $\pm$  S.E.M. of at least three experiments. \*,  $p < 0.01$  compared with control (one-way ANOVA and Dunnett's test).

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**Fig. 5.  $\epsilon$ PKC activation causes translocation of  $\epsilon$ PKC and  $\epsilon$ RACK.** NG108-15/D2 cells were preincubated with or without 1  $\mu$ M  $\epsilon$ PKC specific peptide inhibitor  $\epsilon$ V1-2,  $\delta$ PKC specific inhibitor  $\delta$ V1-1, or conventional PKC specific inhibitor  $\beta$ C2-4 for 30 min respectively. Cells were then incubated with 100 mM ethanol or 50 nM NPA for 10 min. *A*, Detection of  $\epsilon$ PKC, activated  $\epsilon$ PKC (14E6) and  $\epsilon$ RACK translocation was carried out as in Fig. 4. *B*,  $\epsilon$ PKC translocation (green) in 5A was quantitated as the percentage of cells with cytoplasmic staining of  $\epsilon$ PKC. Data are the mean  $\pm$  S.E.M. \*,  $p < 0.01$  compared with control (one-way ANOVA and Dunnett's test).

**Fig. 6.  $\epsilon$ PKC activation and translocation with  $\epsilon$ RACK are separate events.** *A*, NG108-15/D2 cells were pre-treated with or without 20  $\mu$ M Rp-cAMPS and then incubated with the  $\epsilon$ PKC specific agonist, pseudo- $\epsilon$ RACK ( $\psi$  $\epsilon$ RACK, 1  $\mu$ M), for 15 min or with 50 nM NPA for 10 min. *A*, Detection of  $\epsilon$ PKC, activated  $\epsilon$ PKC and  $\epsilon$ RACK translocation was carried out as Fig. 4. *B*, cells were lysed for fractionation. 20  $\mu$ g of cytosolic fraction (C) or particulate fraction (P) was loaded on 10% SDS/PAGE and analyzed for  $\epsilon$ PKC by Western blot. The blot shown is representative of three separate experiments.

**Fig. 7. A model of cross-talk between PKA and PKC signaling.** Scheme represents dopamine/D2- and ethanol/adenosine A2A-induced  $\epsilon$ PKC and  $\epsilon$ RACK translocation. D2

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signaling is indicated by red arrows; ethanol signaling is indicated by blue arrows. D2 activation and ethanol stimulate PLC and increase DAG that activates PKC *via* G $\alpha$ i proteins. Ethanol inhibits adenosine uptake and increases extracellular adenosine that activates G $\alpha$ s-coupled adenosine A2A receptors. D2 activation releases  $\beta\gamma$  that stimulates AC II and IV in the presence of PKC. Both ethanol and D2 activation promote cAMP/PKA signaling. Robust activation of PKA by Sp-cAMPS (black arrow) directly stimulates PLC and activates PKC. Activation of both PKA and PKC leads to the translocation of  $\epsilon$ PKC/ $\epsilon$ RACK complex.

Figure 1

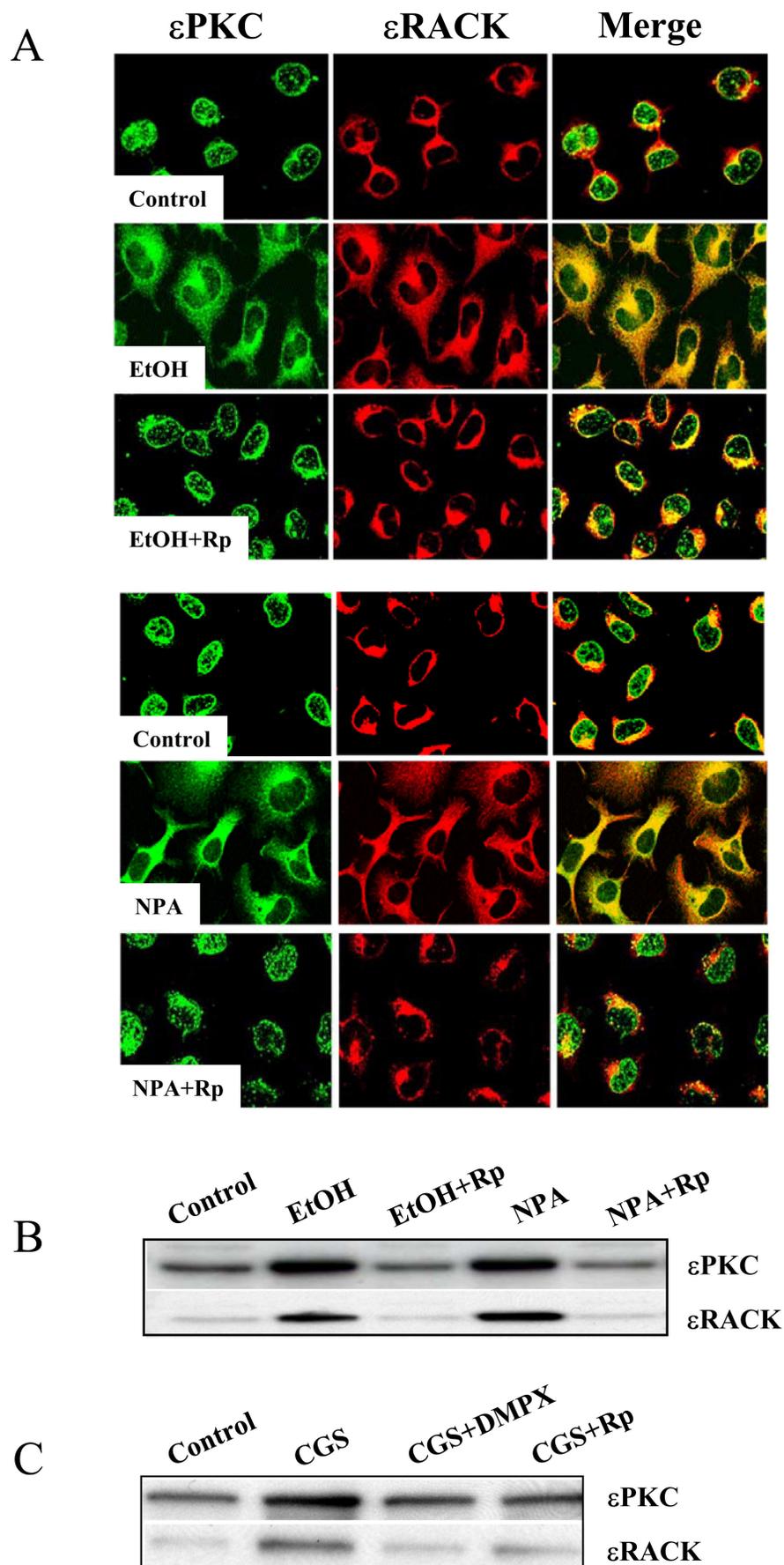


Figure 2

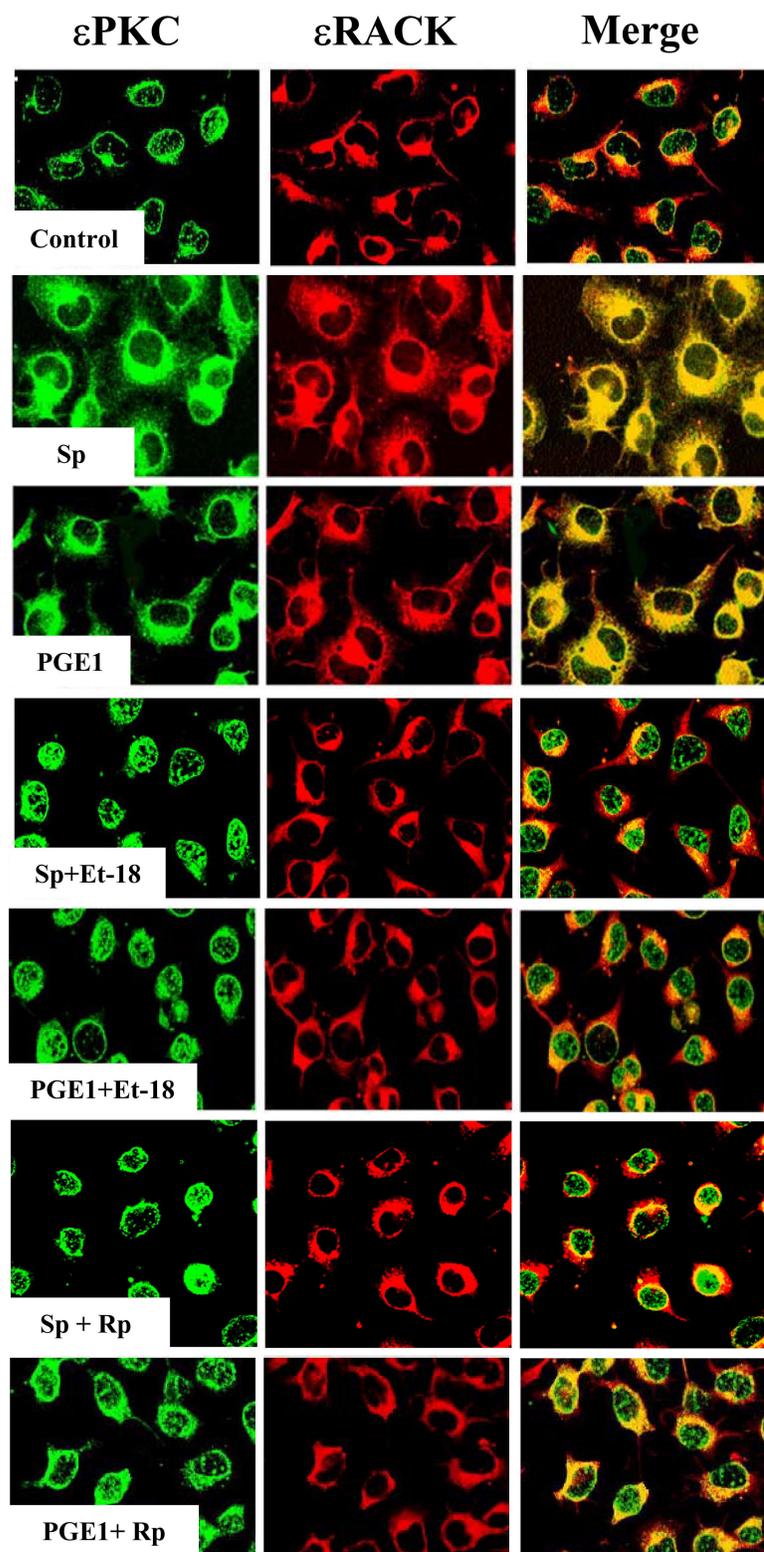


Figure 3

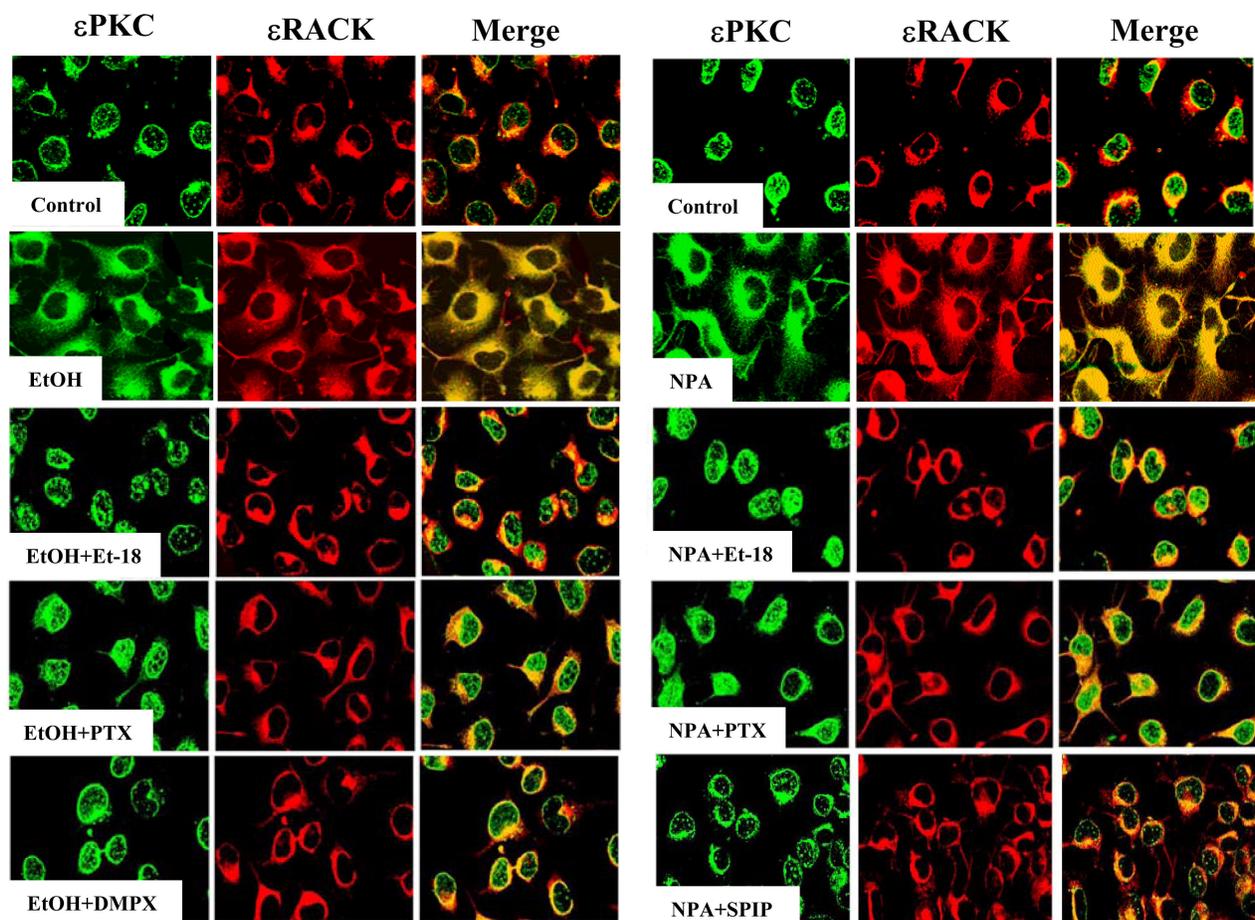
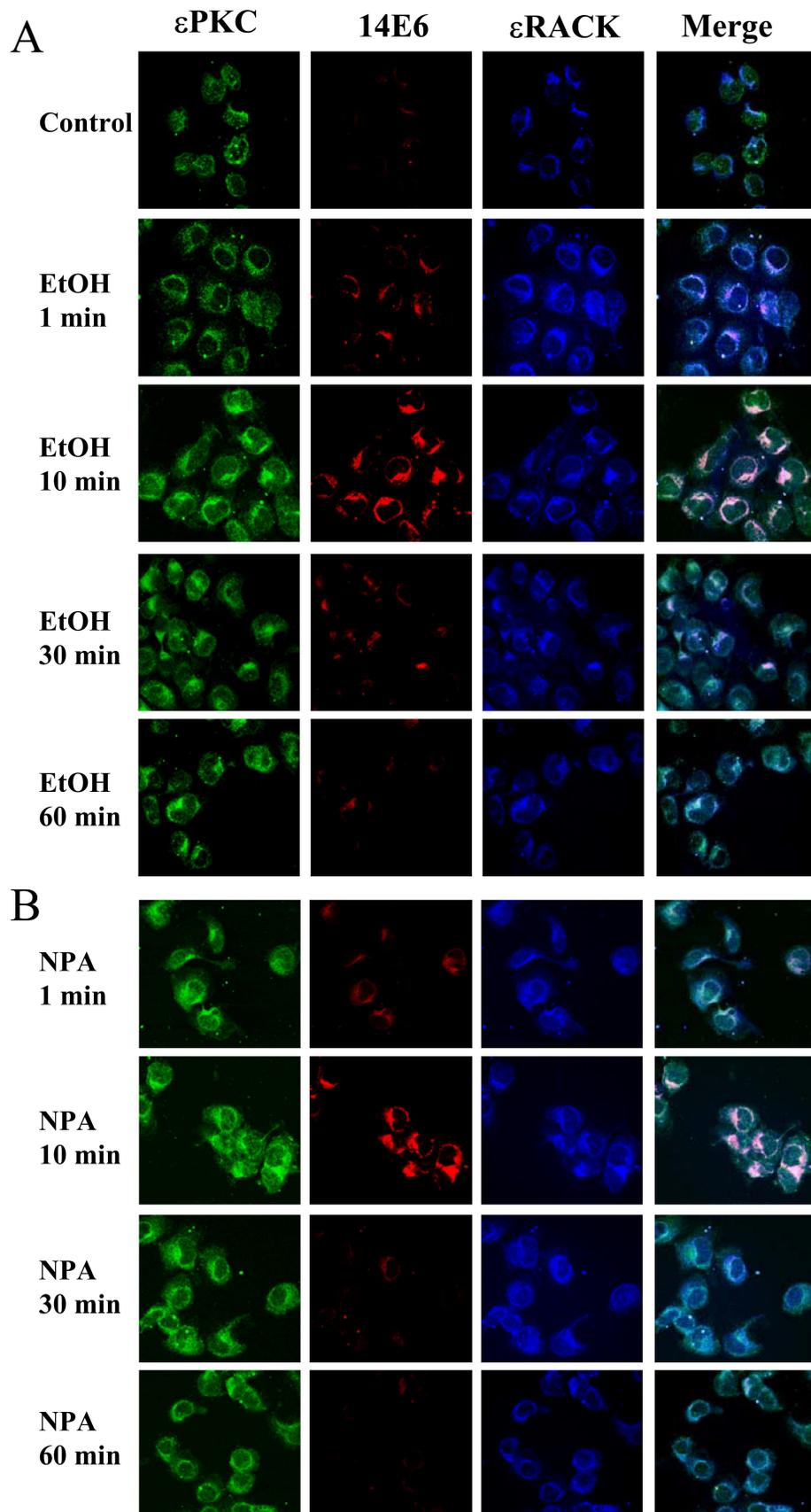


Figure 4



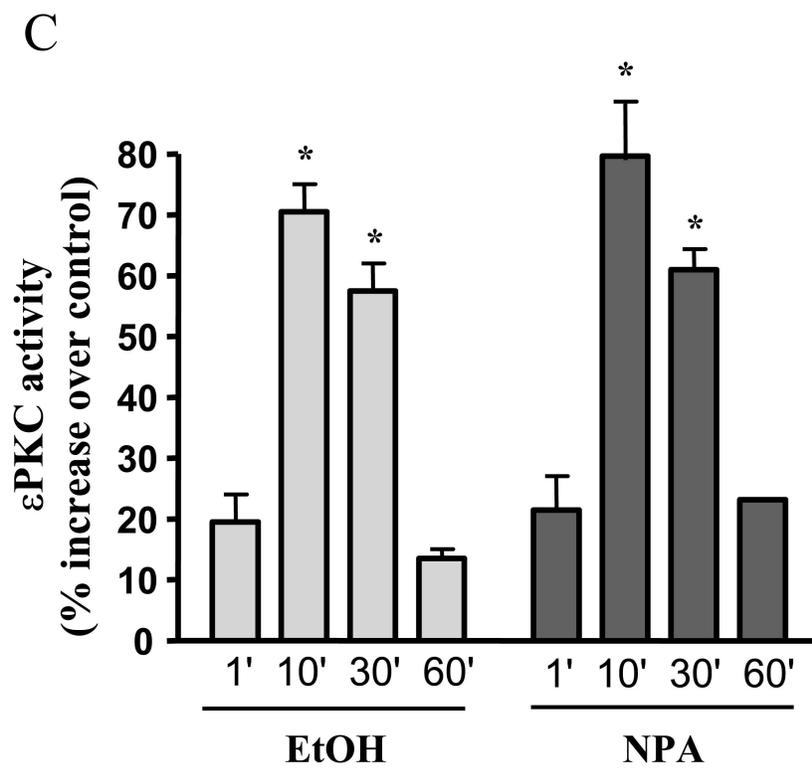


Figure 5

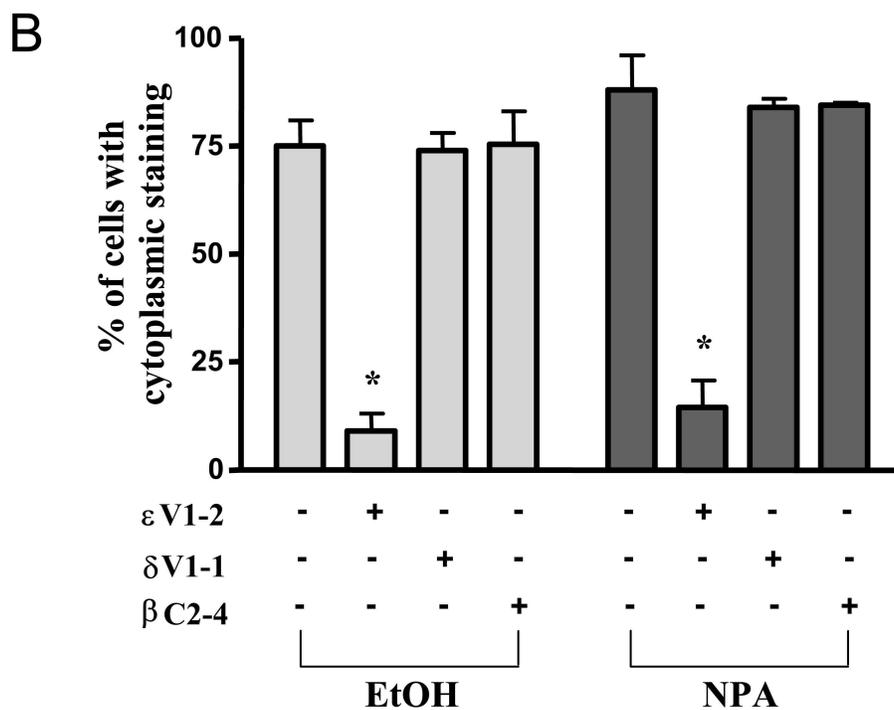
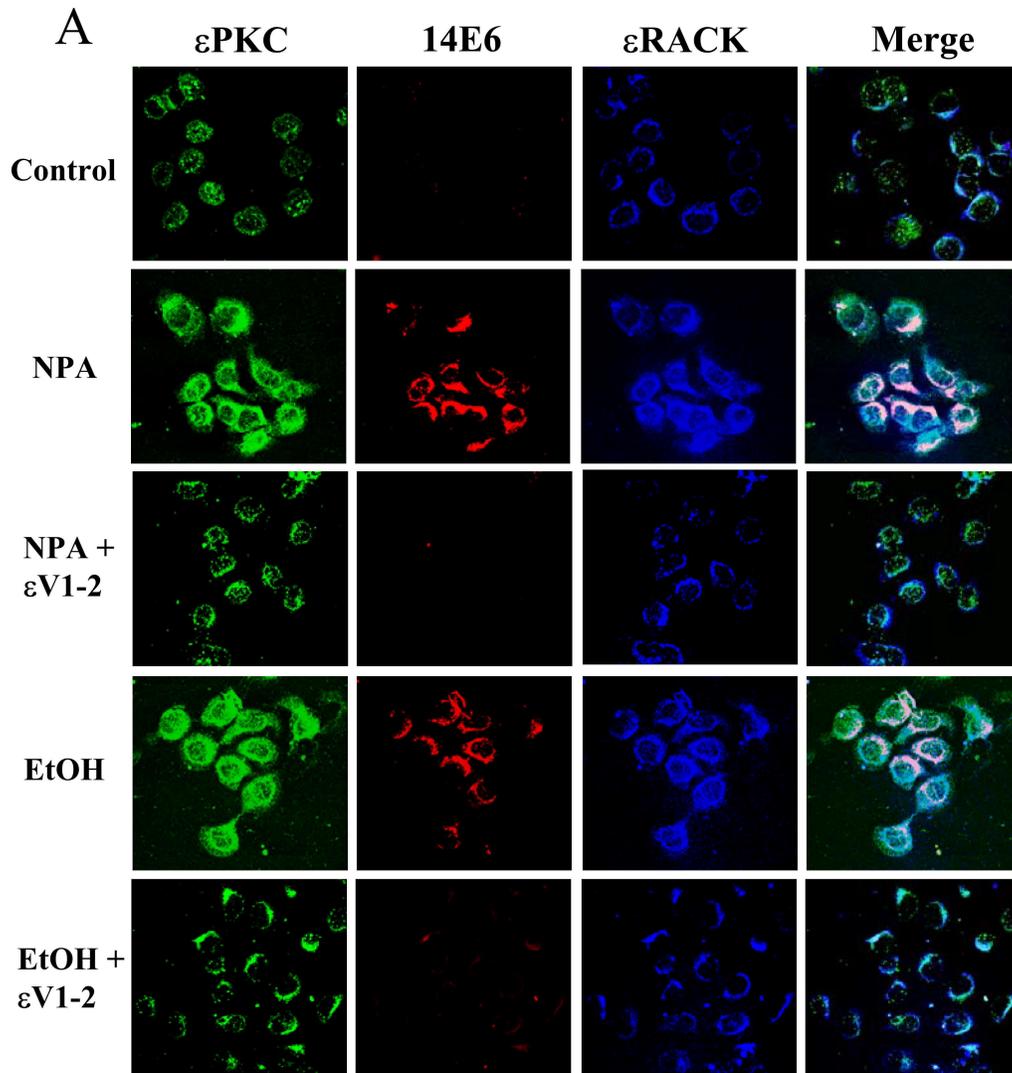


Figure 6

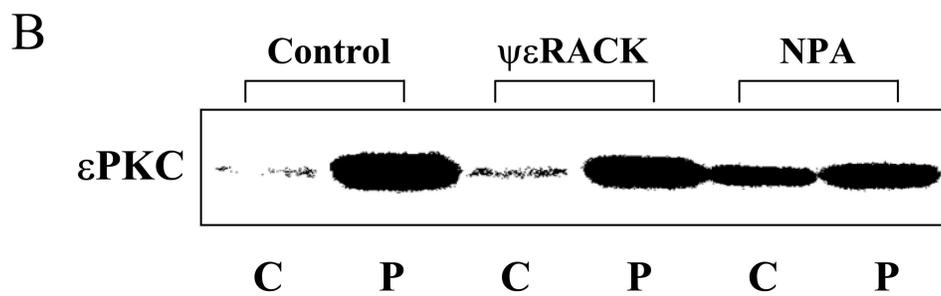
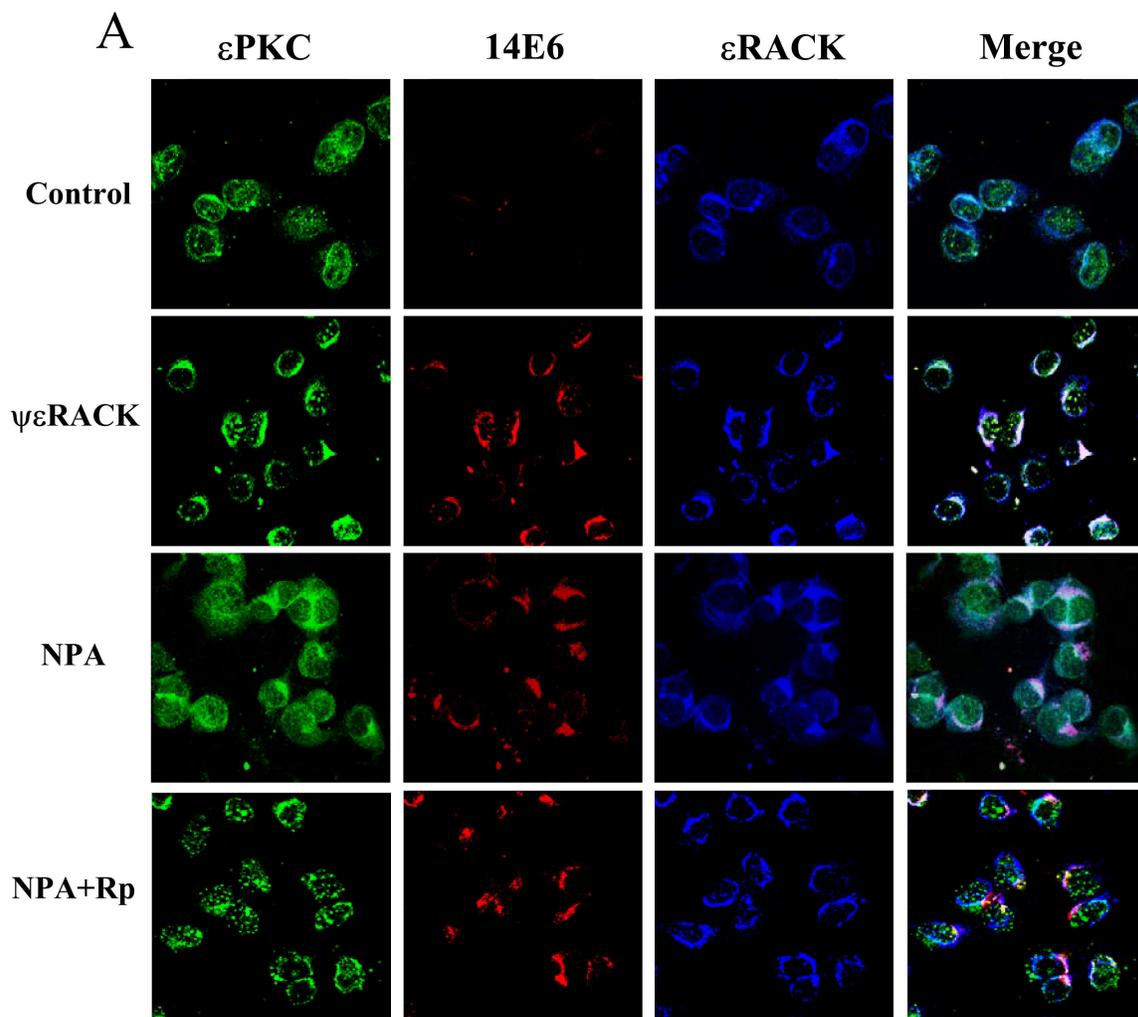


Figure 7

