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# Dopamine and Ethanol Cause Translocation of εPKC Associated with εRACK: Cross-talk Between PKA and PKC Signaling Pathways

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**Running Title:** PKA, PKC, ethanol, dopamine, translocation, cross-talk

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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 (εPKC) and cAMP-dependent protein kinase A (PKA). However, little is known about the mechanism underlying ethanol- and D2-mediated activation of εPKC and the relationship to PKA activation. In the present study, we used a new εPKC antibody, 14E6, that selectively recognizes active εPKC when not bound to its anchoring protein εRACK (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 εPKC and also induced translocation of both εPKC and its anchoring protein, εRACK to a new cytosolic site. The selective εPKC agonist, pseudo-εRACK, activated εPKC but did not cause translocation of the εPKC/εRACK complex to the cytosol. These data suggest a step-wise activation and translocation of εPKC following NPA or ethanol treatment where εPKC first translocates and binds to its RACK and subsequently the εPKC/εRACK complex translocates to a new subcellular site. Direct activation of PKA by Sp-cAMPS, PGE1 or the adenosine A2A receptor is sufficient to cause εPKC 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 εPKC 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 εPKC mediates an intracellular response to ethanol (Gordon et al., 2001; Gordon et al., 1997) and is associated with excessive drinking. εPKC 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 εPKC in the basal forebrain, amygdala, and cerebellum of εPKC 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 εPKC from the perinuclear region to the cytoplasm (Gordon et al., 2001; Gordon et al., 1997). εPKC translocation in ethanol-stimulated cells reached maximum at 30 min, while NPA-induced εPKC 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 εPKC in unstimulated cells (Gordon et al., 2001; Gordon et al., 1997). In this current study, we found that εPKC binding to εRACK precedes its translocation and that PKA is required for the translocation of the εPKC/εRACK 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-OCH3 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 εPKC (mouse IgG raised against the V5 domain of εPKC, Santa Cruz Biotechnology, Santa Cruz, CA), εRACK (rat IgG, Stressgen, Victoria, BC, Canada) for εRACK, and 14E6 (mouse IgM, raised against the V1 domain of εPKC) for active εPKC (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 $MgCL_2$, 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 × 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 εPKC (mouse IgG, Santa Cruz, CA) and εRACK (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 εPKC as described above. To assay εPKC activity, immunoprecipitates were incubated at 30 °C for 20 min with 10 μM ATP, 0.5 μci [$\gamma$-$^{32}$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 εPKC and εRACK to the same location.*** Ethanol and the D2 agonist NPA cause translocation of εPKC (Gordon et al., 2001; Gordon et al., 1997). Activated εPKC associates with the εRACK, β'-COP (Csukai et al., 1997). To determine whether εRACK translocates together with εPKC, 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 εPKC and εRACK. Fig. 1*A* shows that ethanol and NPA both induced εPKC (green) translocation from the nucleus/perinucleus to the cytoplasm, and εRACK (red) from the Golgi/perinucleus to the cytoplasm. The merged images (yellow, Fig. 1*A*) indicate that εPKC and εRACK 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 εPKC in the cytosolic compartment increased concomitantly with the amount of εRACK (Fig. 1*B*), suggesting that εPKC and εRACK moved together after treatment with either ethanol or NPA.

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

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translocation of εPKC and εRACK to the cytoplasm, similar to ethanol and NPA treatments (Fig. 1*A*). 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 εPKC/εRACK, cells were treated with an adenosine A2A agonist CGS21680 for 10 min. We found that CGS21680 mimics ethanol-induced translocation of εPKC/εRACK (Fig. 1*C*). This translocation was blocked by the A2A antagonist DMPX or the PKA inhibitor Rp-cAMPS (Fig. 1*C*). Rp-cAMPS and DMPX had no effect on the localization of εPKC and εRACK 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 εPKC *via* PLC in NG108-15/D2 cells. We found that the PLC inhibitor, Et-18-OCH3 without effect in unstimulated cells (data not shown), inhibits Sp-cAMPS- or PGE1-induced translocation of εPKC and εRACK (Fig. 2). As expected, Rp-cAMPS also prevented these translocations (Fig. 2).

***Ethanol and NPA cause translocation of εPKC/εRACK complex via the PLC/PKC system.*** We have previously shown that ethanol- or NPA-induced translocation of εPKC is blocked by the PLC inhibitor Et-18-OCH3 (Gordon et al., 2001). If PLC activation is required for ethanol- and NPA-induced translocation of εPKC, then inhibition of PLC activity should also inhibit translocation of εPKC/εRACK. Indeed, the PLC inhibitor Et-

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18-OCH3 blocked εRACK translocation along with εPKC (Fig. 2 and 3). As anticipated, the PKC inhibitor GF 109203X also blocked translocation (data not shown).

Because both Gα and βγ released from trimeric G proteins can stimulate PLCβ isozymes (Camps et al., 1992; Park et al., 1993; Runnels and Scarlata, 1999) and PTX inhibits ethanol- and NPA-induced translocation of εPKC (Gordon et al., 2001), we next asked whether εRACK translocation requires Gαi. We found that PTX, which inhibits Gαi/o and βγ, prevented co-translocation of εRACK with εPKC (Fig. 3). We know that the A2A antagonist DMPX blocks ethanol- but not NPA-induced εPKC translocation and that the D2 antagonist spiperone blocks NPA- but not ethanol-induced εPKC translocation (Gordon et al., 2001). Here, we show that DMPX or spiperone each prevents ethanol- or NPA-induced εRACK translocation separately (Fig. 3). In contrast, PTX, DMPX or spiperone alone was without effect on the localization of εPKC and εRACK in unstimulated cells (data not shown). Taken together, these findings suggest that ethanol, *via* the adenosine A2A receptor, and dopamine, *via* the D2 receptor, cause εPKC and εRACK translocation by stimulating the PLC/PKC signaling pathway.

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

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

To determine which PKC isozymes mediate ethanol- or NPA-induced translocation of εPKC and εRACK, cells were treated with isozyme-selective translocation peptide inhibitors: εV1-2 for εPKC, δV1-1 for δPKC or βC2-4 for conventional PKC (Schechtman and Mochly-Rosen, 2002) respectively, prior to the treatment of ethanol or NPA. We found that εV1-2, but not δV1-1 or βC2-4, prevented ethanol- or NPA-induced translocation of εPKC and εRACK (Fig. 5*A,B*). In control experiments, these peptide inhibitors did not alter the localization of εPKC and εRACK in unstimulated cells (data not shown). These results suggest that εPKC activation is solely responsible for ethanol- and NPA-induced translocation of εPKC and εRACK.

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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. 6*A*). Cells treated with NPA, which stimulates both PKA and PKC, showed translocation of εPKC and εRACK to this cytoplasmic compartment (Fig. 6*A*). 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. 6*A*). Western blot analysis confirmed that NPA, but not pseudo-εRACK, caused εPKC translocation from the particulate to the cytosolic fraction (Fig. 6*B*). 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, εPKC translocates from the perinucleus/nucleus to a new perinuclear/Golgi compartment, perhaps where εRACK is colocalized in unstimulated cells. Subsequently, εPKC and the εRACK translocate from the perinucleus/Golgi to the cytosol. Translocation of εPKC and εRACK to the cytosol occurs only when PKA is activated, a process that is Gαi-dependent. Consistent with this observation, the εPKC agonist, pseudo-εRACK, did not cause the translocation of εPKC to the cytosol although it activated εPKC. Moreover, activation of PKA by Sp-cAMPS, PGE1 or the adenosine A2A receptor alone is sufficient to cause εPKC and εRACK translocation. Importantly, PKA-dependent translocation of εPKC was inhibited by the PLC inhibitor Et-18-OCH3, 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 εPKC/εRACK 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 (Cα) 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 Cα 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 Cα translocation is likely due to βγ activation of adenylyl cyclase (AC) II and/or IV, because PTX and βγ scavenger peptide prevent PKA Cα translocation (Yao et al., 2002; Yao et al., 2003). βγ activation of AC II or IV requires either Gαs (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 Gαs and βγ, respectively. Moreover, the PKC inhibitor bisindolylmaleimide I (GF 109203X) blocks PKA Cα translocation induced by NPA, but not by ethanol (Yao at al., unpublished observation).

**PKC and RACK.** Ethanol and NPA also induce translocation of εPKC. 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 εPKC activation and translocation. Using the antibody 14E6, we show that ethanol and NPA activate εPKC and increase the catalytic activity of εPKC measured by phosphorylation. We also show that εV1-2, an inhibitor of εPKC binding to its RACK, prevents ethanol- and NPA-induced translocation of the εPKC/εRACK complex. In contrast, the peptide inhibitor δV1-1 (δPKC) or βC2-4 (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 εPKC. The activated εPKC binds first to its RACK and subsequently translocates from the perinucleus to a new cytoplasmic compartment. Thus, activation of εPKC appears to be necessary for εPKC and εRACK translocation (Fig. 7). However, activation of εPKC alone is not sufficient to cause translocation of the εPKC/εRACK complex because the εPKC agonist, pseudo-εRACK, does not cause translocation of εPKC into the cytoplasm despite activating εPKC. These observations demonstrate that PKA activation induced by ethanol or NPA has a dual role in εPKC signaling: first PKA activates PLC to produce DAG for εPKC activation and second, PKA causes relocation of activated εPKC/εRACK. This is likely to yield different cellular responses, as the protein substrates of εPKC 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 Gαs, increases PKC activity and translocation in LTK cells (Yu et al., 1996). In this study, we demonstrate that **translocation** of εPKC and εRACK by ethanol and NPA requires PKA activation but the PKA inhibitor Rp-cAMPS does not

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inhibit the **activation** of εPKC. These findings suggest that PKA may regulate the location of εPKC/εRACK complex while not affecting the activation state of the enzyme (Fig. 7). Indeed, prosite analysis reveals a consensus PKA phosphorylation site in εRACK (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 εPKC and εRACK. Importantly, direct activation of the adenosine A2A receptor by CGS21680 also caused translocation of εPKC/εRACK to the same compartment. We propose that activation of PKA stimulates PLCβ, thus increasing DAG levels and causing activation and translocation of εPKC (Fig. 7). Indeed, a PLC inhibitor blocks εPKC translocation. However, it is still unclear how ethanol, NPA or PKA activate the correct pool of PKC and how activated εPKC translocates with εRACK 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, εRACK, β'-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, εPKC and εRACK (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, εPKC, and εRACK, and how AKAP targets PKA and εPKC 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 εPKC appears to play a role in drinking behaviors; mice lacking εPKC 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 εPKC and εRACK 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 μM) for 20 min before the treatment of ethanol or NPA. Cells were fixed and stained for εPKC (green, mouse IgG against the V5 domain of εPKC, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and εRACK (red), and scanned using a Bio-Rad 1024 confocal microscope. The merged images of εPKC with εRACK 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 εPKC or a monoclonal εRACK antibody. *C*, Cells were preincubated with or without the PKA inhibitor, Rp-cAMPS (Rp, 20 μM) or the adenosine A2A antagonist, DMPX (10 μM) for 20 min and then treated with the A2A agonist, CGS21680 (CGS, 100 nM) for 10 min. Immunoprecipitation and detection of εPKC/εRACK translocation were performed as *B*.

**Fig. 2. PKA stimulates PLC to induce εPKC and εRACK translocation.** NG108–15/D2 cells were preincubated with or without the PLC inhibitor, Et-18-OCH3 (Et-18, 10

μM) or the PKA inhibitor, Rp-cAMPS (Rp, 20 μM) for 20 min and then treated with 1 mM Sp-cAMPS (Sp) or 10 μM PGE1 for 10 min. Analyses of εPKC and εRACK translocation were performed as Fig. 1*A*.

**Fig. 3. Ethanol- and NPA- induced εPKC and εRACK translocation requires PLC.** NG108–15/D2 cells were preincubated with or without the PLC inhibitor Et-18 (10 μM), the A2A antagonist DMPX (10 μM), or the D2 antagonist spiperone (SPIP, 10 μM) 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 εPKC and εRACK translocation were carried out as Fig. 1*A*.

**Fig. 4. εPKC and εRACK 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 εPKC (green), activated εPKC (14E6, red) and εRACK (blue). The merged images were produced using anti-mouse IgG against the V5 domain of εPKC (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), state-specific anti-εPKC antibodies, 14E6, and anti-εRACK antibodies. *C*, Cells were lysed and immunoprecipitated with anti-mouse εPKC 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 ± 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. εPKC activation causes translocation of εPKC and εRACK.** NG108-15/D2 cells were preincubated with or without 1 μM εPKC specific peptide inhibitor εV1-2, δPKC specific inhibitor δV1-1, or conventional PKC specific inhibitor βC2-4 for 30 min respectively. Cells were then incubated with 100 mM ethanol or 50 nM NPA for 10 min. *A*, Detection of εPKC, activated εPKC (14E6) and εRACK translocation was carried out as in Fig. 4. *B*, εPKC translocation (green) in 5*A* was quantitated as the percentage of cells with cytoplasmic staining of εPKC. Data are the mean ± S.E.M. *, $p < 0.01$ compared with control (one-way ANOVA and Dunnett's test).

**Fig. 6. εPKC activation and translocation with εRACK are separate events.** *A*, NG108-15/D2 cells were pre-treated with or without 20 μM Rp-cAMPS and then incubated with the εPKC specific agonist, pseudo-εRACK (ψεRACK, 1 μM), for 15 min or with 50 nM NPA for 10 min. *A*, Detection of εPKC, activated εPKC and εRACK translocation was carried out as Fig. 4. *B*, cells were lysed for fractionation. 20 μg of cytosolic fraction (C) or particulate fraction (P) was loaded on 10% SDS/PAGE and analyzed for εPKC 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 εPKC and εRACK 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* Gαi proteins. Ethanol inhibits adenosine uptake and increases extracellular adenosine that activates Gαs-coupled adenosine A2A receptors. D2 activation releases βγ 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 εPKC/εRACK complex.

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Figure 1

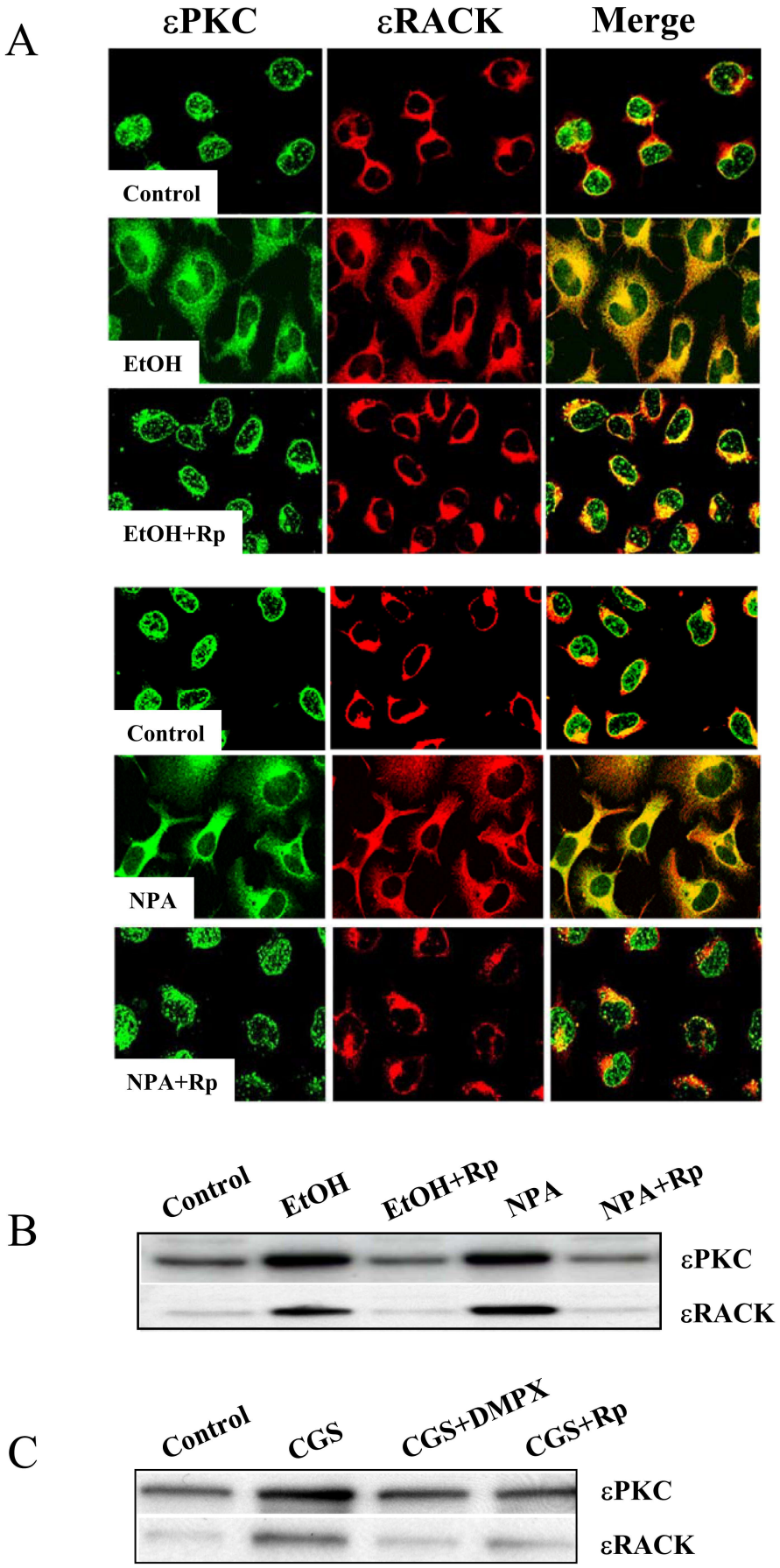

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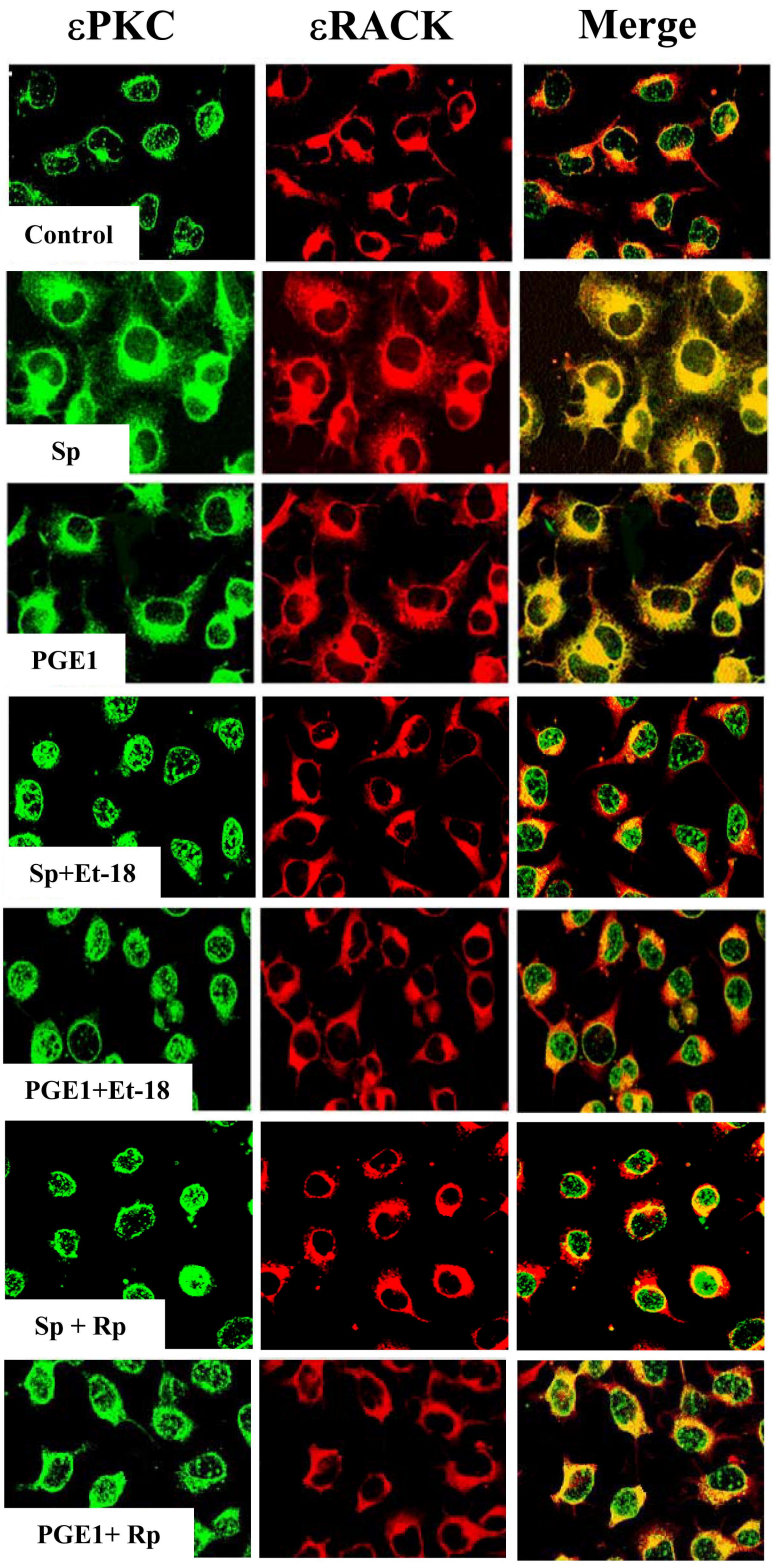
Figure 2



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# Figure 3

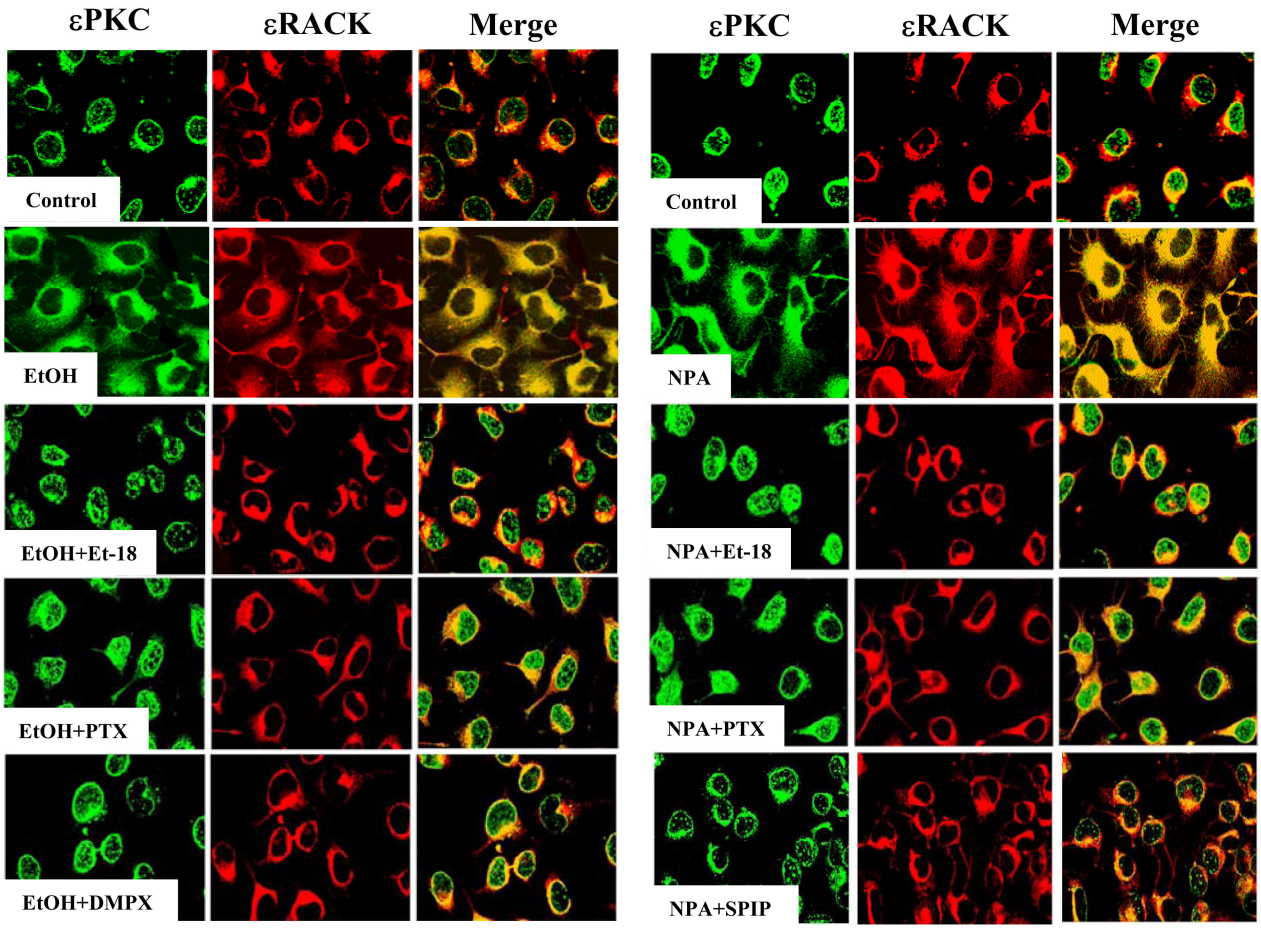

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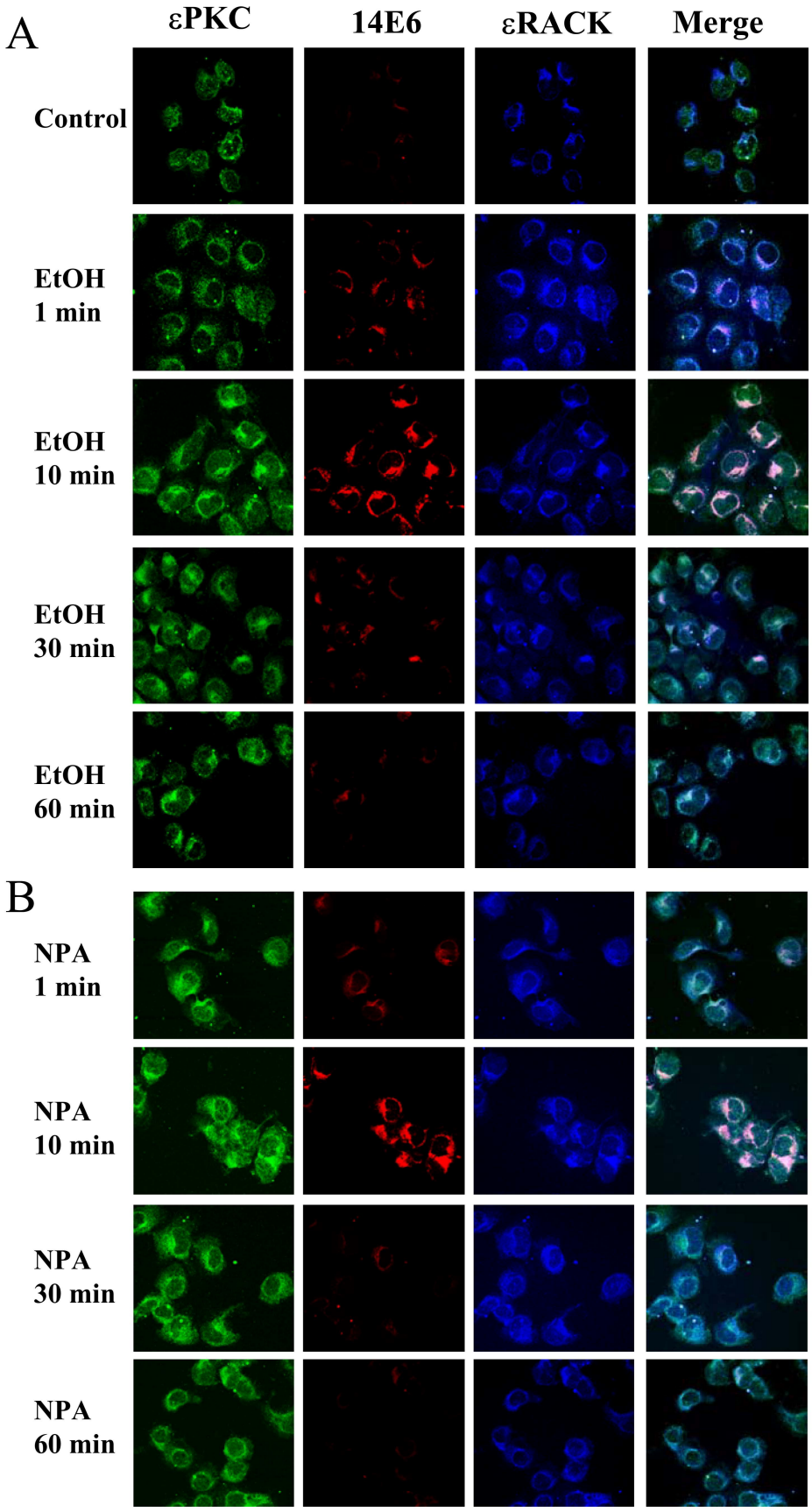
Figure 4



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C

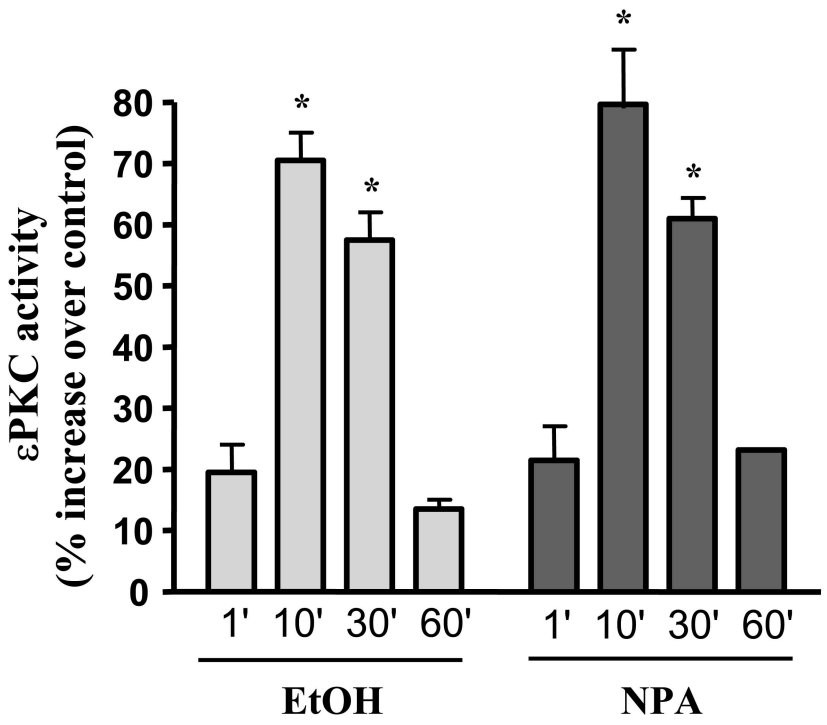

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Figure 5

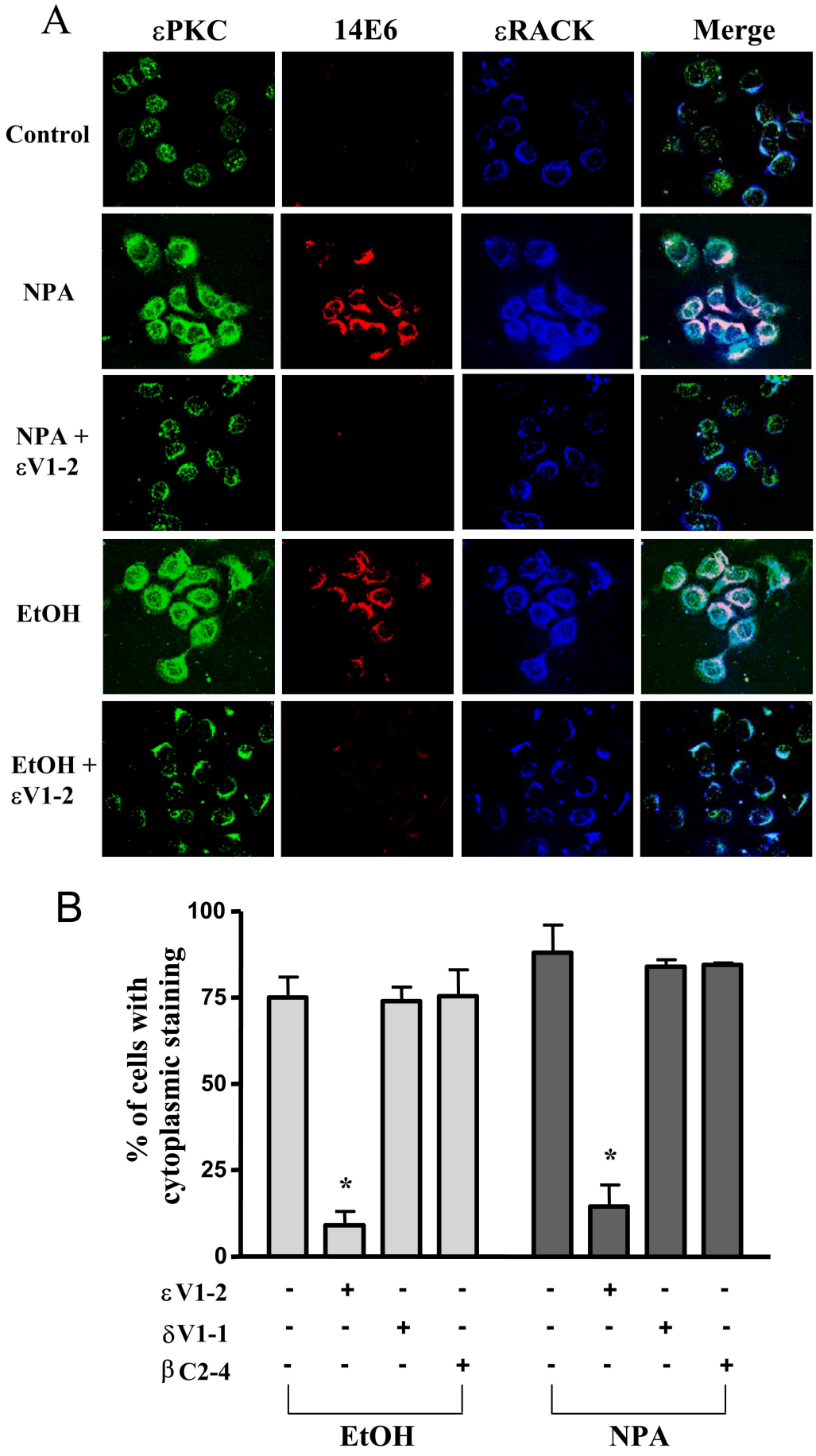

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Figure 6

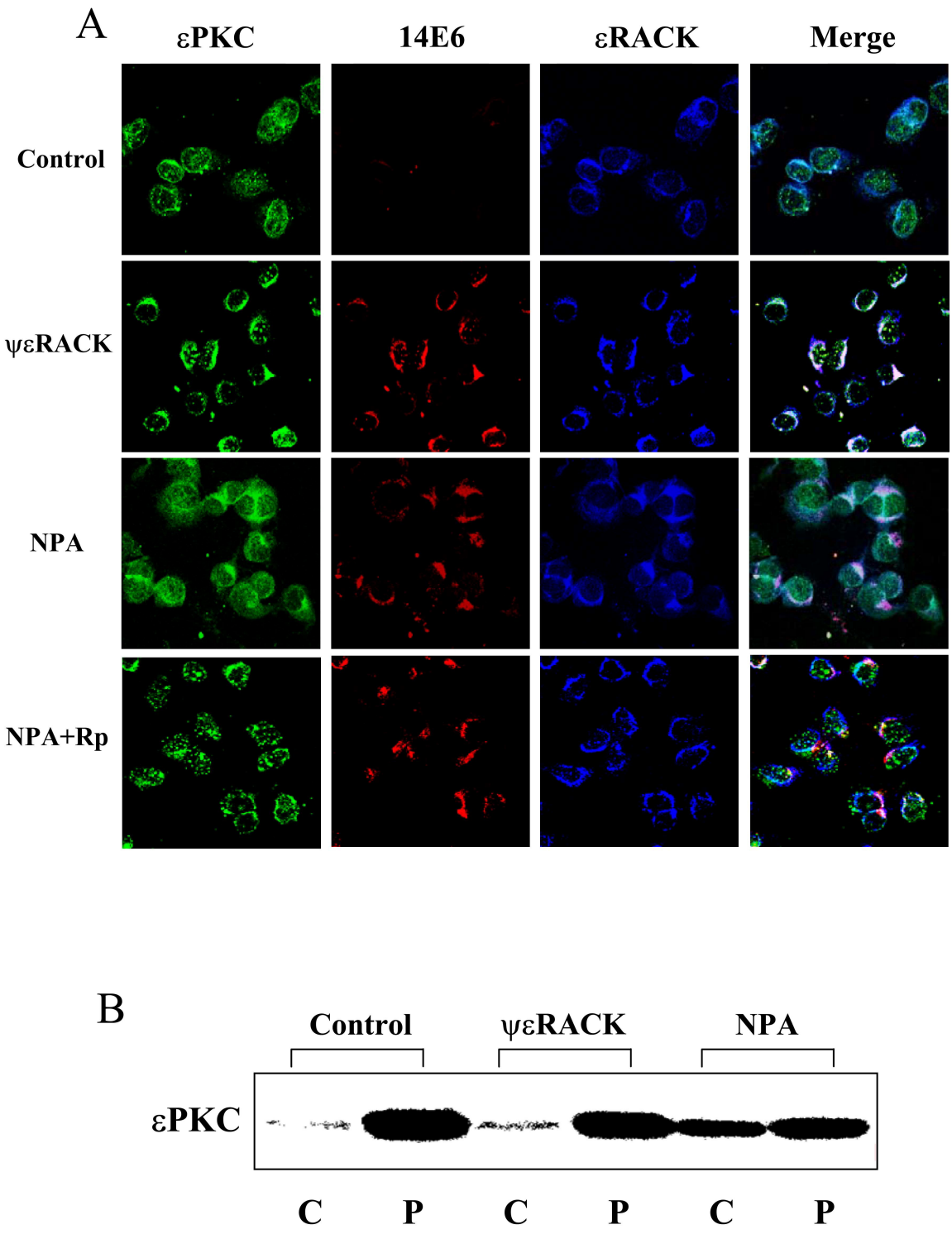

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Figure 7