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Ethanol Acts Synergistically with a D2 Dopamine Agonist to Cause Translocation of Protein Kinase C

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ABSTRACT

Ethanol and other drugs of abuse increase synaptic dopamine levels; however, little is known about how ethanol alters dopaminergic signaling. We have reported that ethanol induces translocation of δ and ϵ protein kinase C (PKC) in neural cells in culture. Using NG108–15 and Chinese hamster ovary cell lines that express the dopamine D2 receptor (D2R), we show here that the D2R agonist *R*(−)-2,10,11-trihydroxy-*N*-propyl-noraporphine hydrobromide (NPA) also causes translocation of δ and ϵ PKC to the same sites as ethanol-induced translocation. D2R agonist and ethanol-induced translocation of δ and ϵ PKC share a common pathway that is blocked by pertussis toxin and requires phospholipase C (PLC) activity. These data suggest that both D2R agonists and ethanol activate PLC via a trimeric G protein leading to production of diacylglycerol with subsequent activation and translocation of δ and ϵ PKC. Moreover, ethanol and NPA, when present together at low concentrations that alone are ineffective, act synergistically to cause translocation of δ and ϵ PKC. Our data suggest that ethanol causes translocation of δ and ϵ PKC but cells expressing the D2R, such as neurons in the nucleus accumbens, may be particularly sensitive to low concentrations of ethanol.

Our laboratory has shown that ethanol causes translocation of the δ and ϵ isoforms of protein kinase C (PKC) in NG108–15 cells (Gordon et al., 1997). These isozymes remain translocated as long as ethanol is present and return to their original sites only after ethanol is withdrawn (Gordon et al., 1997). Ethanol and other addictive drugs increase dopamine levels in the nucleus accumbens (Weiss et al., 1993; Self and Nestler, 1995; Samson and Hodge, 1996) and the D2 dopamine receptor (D2R), as well as other members of the dopamine receptor family [e.g., D1, D3 and D4 receptors (Rubinstein et al., 1997; Pilla et al., 1999)], have been shown to participate in many of the behaviors related to drugs of abuse (Hodge et al., 1996; Maldonado et al., 1997; Phillips et al., 1998; Volkow et al., 1999). Varying effects of dopamine receptor activation on the PKC signal transduction pathway have been reported [for review, see Missale et al. (1998)]. However, little is known about dopamine receptor regulation of PKC in individual neurons or about ethanol-induced changes in PKC activity in neurons expressing the D2R. We have undertaken a study in cultured cell lines to determine whether dopamine signaling via the D2R also causes translocation of δ and ϵ PKC and, most importantly, whether ethanol and D2R agonists act synergistically in a single neural cell. Here, we present evidence that the D2R agonist, *R*(−)–2,10,11-trihydroxy-*N*-propyl-noraporphine hydrobromide (NPA), causes translocation of δ and ϵ PKC. Furthermore, we find that ethanol mimics D2R activation and that ethanol and NPA can act synergistically to induce translocation of these PKC isozymes. These results suggest a mechanism that may underlie the regulation of cellular functions by ethanol, particularly in dopaminergic pathways that are thought to be involved in craving and reward. Neurons expressing the D2R, such as those in the nucleus accumbens, may be selectively activated by low concentrations of ethanol.

Experimental Procedures

Materials. All reagents were purchased from Sigma (St. Louis, MO) except where indicated. Ham's F-12 medium was purchased from Life Technologies (Grand Island, NY), and NPA and spiperone

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ABBREVIATIONS: PKC, protein kinase C; D2R, dopamine D2 receptor; NPA, *R*(−)-2,10,11-trihydroxy-*N*-propyl-noraporphine hydrobromide; NG108–15/D2, NG108–15 cells stably expressing the dopamine D2 receptor; CHO/D2, Chinese hamster ovary cells stably expressing the dopamine D2 receptor; PLC, phospholipase C; DAG, diacylglycerol; PTX, pertussis toxin; RACK, receptors for activated PKC.

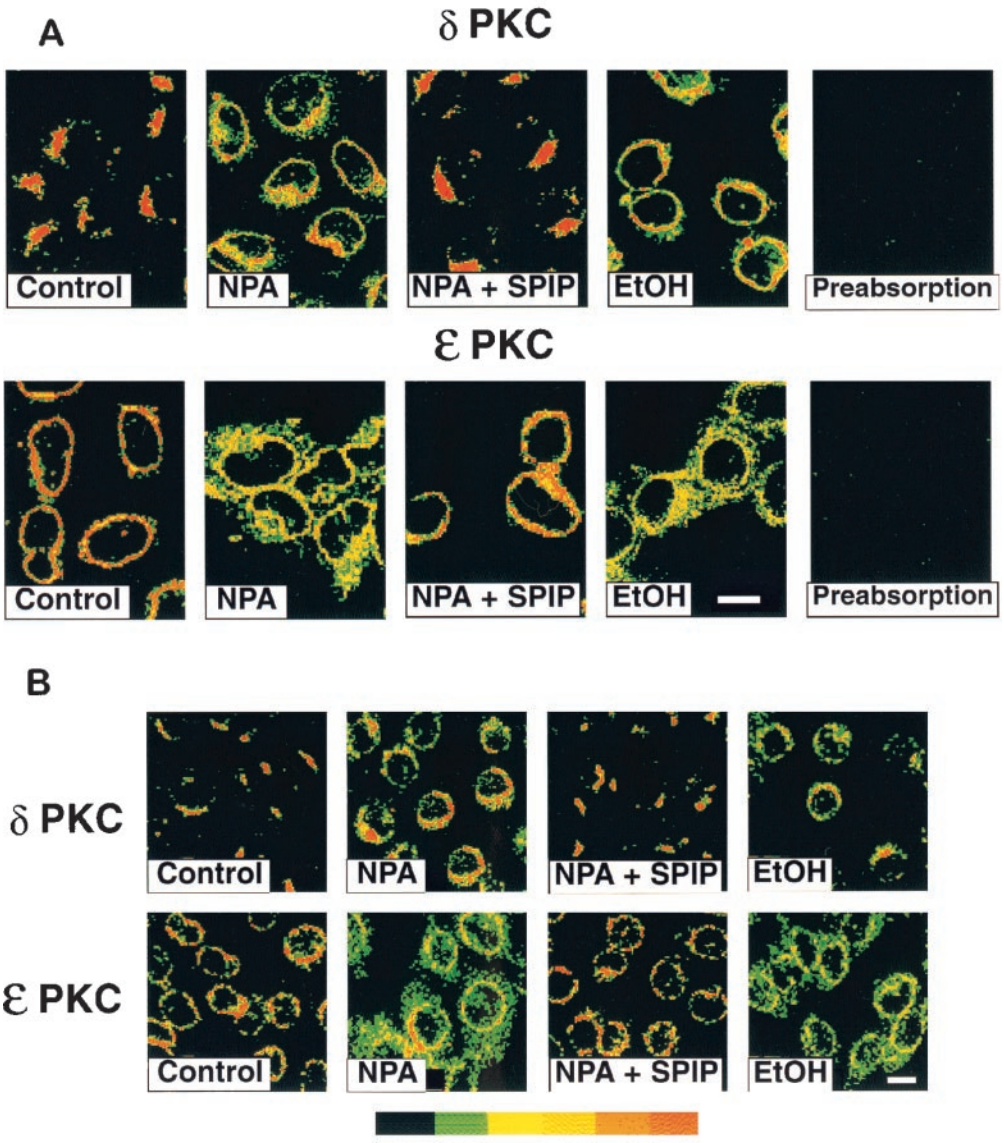

Fig. 1. Ethanol and NPA, a dopamine D2 receptor agonist, cause translocation of δ and ϵ protein kinase C in NG108–15/D2 and CHO/D2 cells. A, NG108/15 cells expressing the D2R were exposed to 200 mM ethanol or the D2R-specific agonist, NPA (50 nM), for 30 min. Where indicated, the cells were preincubated with the dopamine receptor antagonist spiperone (SPIP; 10 μM) for 20 min before the addition of NPA and during incubation with NPA. Cells were fixed and stained for δ and ϵ PKC as described under *Experimental Procedures* and scanned using a Bio-Rad 1024 confocal microscope. The data are representative of at least three independent experiments. Preabsorption of the isozyme-specific antibody with the respective peptide antigens, as described in Gordon et al. (1997), specifically blocked immunostaining by each antibody. Staining intensity is indicated by the color bar: orange indicates areas of most intense staining. (Scale bar, 10 μm) (Images are 400×.) B, CHO/D2 cells treated as in A above.

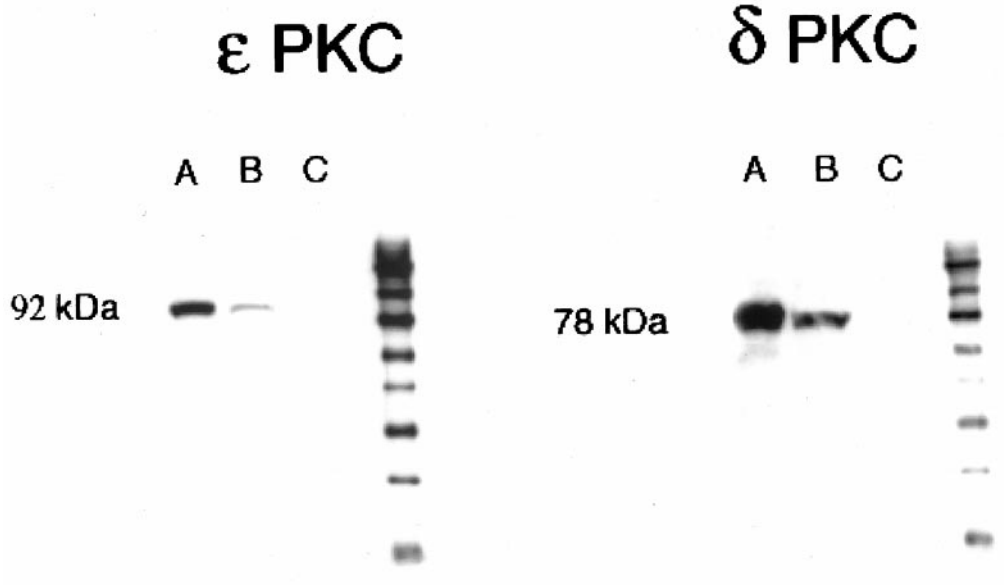

Fig. 2. Isozyme specificity of anti-δ and -ϵ antibodies. Western blot of wild type (WT) mouse whole brain extracts (20 μg of protein) (A); NG105/D2 whole cell extracts (20 μg of protein) (B); or −/− ϵ PKC or −/− δ PKC mouse whole brain extracts (20 μg of protein) (C) probed with either anti-ϵ PKC antibodies (left) or anti-δ PKC antibodies (right).

were purchased from RBI (Natick, MA). U73122 and Et-18-OCH3 were purchased from Calbiochem (San Diego, CA). BODIPY TR ceramide was purchased from Molecular Probes (Eugene, OR).

Cell Culture. NG108–15 cells stably expressing the rat D2L receptor (NG108–15/D2;15 fmol/mg of protein) (Asai et al., 1998) were grown on single-well slides in defined media for 3 days (Dohrman et al., 1996). Beginning 48 h after plating, media were replaced daily with defined media. On day 4, the cells were treated as described in the figure legends and fixed as described below (Gordon et al., 1997). Chinese hamster ovary cells stably expressing the murine D2L receptor (CHO/D2; 1–3 pmol/mg of protein) (Fishburn et al., 1995) were grown in single-well slides in 10% fetal bovine serum: Ham's F-12 (1:1); media were replaced daily.

Immunocytochemistry. Cells were fixed with cold methanol for 2 to 3 min. Slides were 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, and primary antibodies specific for δ or ϵ PKC (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted 1:150 and 1:100, respectively, and, where indicated, the Golgi marker BODIPY TR ceramide. The cells were then washed three times with PBS, incubated for 1 h at room temperature with fluorescein isothiocyanate-conjugated anti-rabbit secondary antibody (Cappel, Aurora, OH) (diluted 1:1000) and washed again three times with PBS and coverslipped using Vectashield mounting medium.

Microscopy. 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. Collected data were processed using NIH

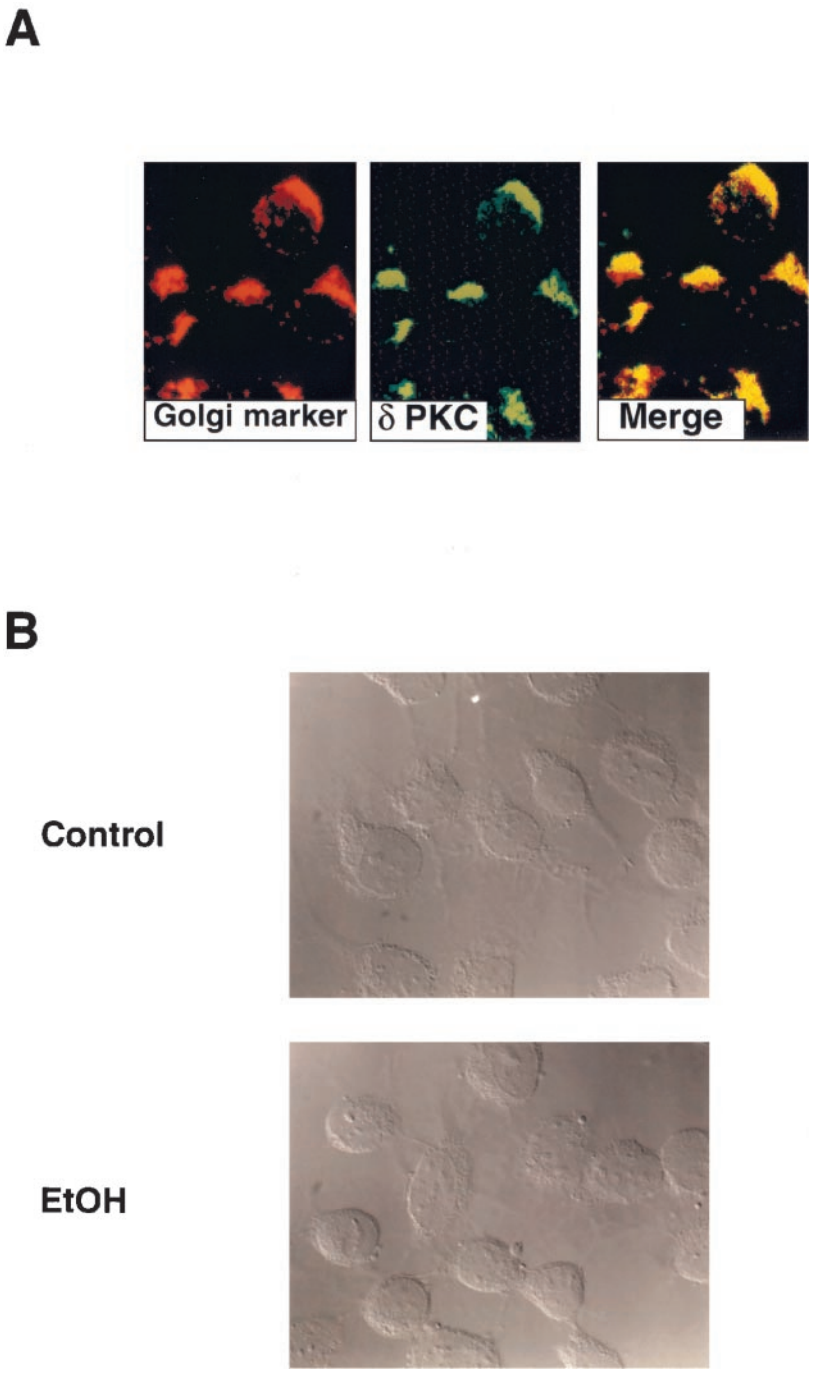

Fig. 3. Co-localization of a Golgi marker with δ PKC and morphology of NG108–15 wild-type cells. A, double-labeling for the Golgi area and δ PKC in control NG108–15/D2 cells. Cells were fixed and stained as described above for δPKC (green) and the Golgi marker BODIPY TR ceramide (red). Orange color indicates colocalization of δ PKC and the Golgi. Images are obtained with a BioRad 1024 scanning laser confocal microscope and are 400×. B, wild-type NG108–15 cells were incubated for 30 min in the absence or presence of 200 mM ethanol. Images (400×) were obtained using Nomarski (DIC) optics.

Image and Adobe Photoshop software (Adobe, Mountain View, CA). Images shown are a single plane near the center of the cell. Nomarski (DIC) images (Fig. 2B) were taken using a Leica DMRD microscope with a Spot camera and processed using Adobe Photoshop software.

Quantification of PKC Localization. For quantification of ϵ-PKC translocation (Fig. 4), random fields on each slide were selected and cells scored for perinuclear staining or cytoplasmic staining (defined as the number of cells possessing staining of an area in the cytoplasm at a distance greater than the radius of the nucleus). At least four fields were scored for each experiment, for a total number of at least 50 cells per slide. Data shown were obtained by two independent observers who were blind to the experimental condition.

Results

A D2R Agonist Induces Translocation of δ and ϵ PKC. NG108–15 cells were transfected with plasmids encoding the cDNA for the long form of the D2R and a neomycin resistance gene, and a stable cell line was selected (NG108–15/D2 cells) (Asai et al., 1998). In control cells, δ PKC appeared to localize to the Golgi area (Fig. 1A); this was confirmed by colocalization of δ PKC with the Golgi marker BODIPY TR ceramide (Fig. 3A). ϵ PKC in control cells was localized to the perinucleus (Fig. 1A). When NG108–15/D2 cells were incubated for 30 min with the D2R agonist NPA, δ PKC translocated from the Golgi area primarily to the perinucleus but also to the nucleus, and ϵ PKC translocated from the perinucleus to the cytoplasm (Fig. 1A). The dopamine receptor antagonist spiperone blocked NPA-induced δ and ϵ PKC translocation (Fig. 1A). As expected, NPA did not affect δ or ϵ PKC localization in wild-type NG108–15 cells, which lack the D2 receptor (data not shown). Ethanol alone also caused translocation of δ and ϵ PKC to the perinucleus and cytoplasm, respectively (Fig. 1A), as reported previously (Gordon et al., 1997). Spiperone did not block ethanol-induced translocation of either isozyme (data not shown).

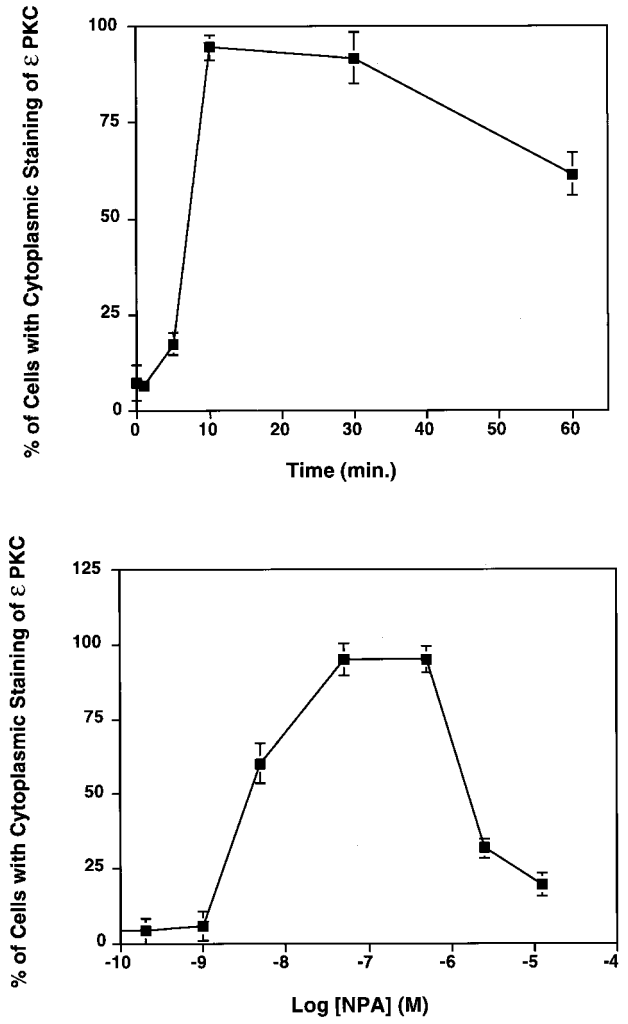

Fig. 4. Translocation of ϵ PKC is dependent on time and concentration. A, ϵ PKC translocation induced by 50 nM NPA in CHO/D2 cells as a function of time. CHO/D2 cells were incubated with 50 nM NPA for the indicated times, and the percentage of cells with cytoplasmic staining of ϵ PKC (defined as the number of cells possessing staining of an area in the cytoplasm greater than the radius of the nucleus) divided by the total number of cells was calculated. Data are the mean ± S.E.M. (four fields per experiment for a total number of at least 50 cells per slide, $n = 3$). B, ϵ PKC translocation in CHO/D2 cells as a function of NPA concentration. CHO/D2 cells were incubated with the indicated concentrations of NPA for 30 min and the percentage of cells with cytoplasmic staining of ϵ PKC determined as in A. Data are the mean ± S.E.M. (four fields per experiment for a total number of at least 50 cells per slide, $n = 3$).

We confirmed the isozyme specificity of the antibodies for the immunogenic peptides by preabsorption with the respective peptides (Fig. 1A). The isozyme selectivity of the antibodies we used for δ PKC and ϵ PKC was determined using brain extracts from wild-type mice and from mice with specific deletions of δ PKC (Michael Leitges, personal communication) and ϵ PKC (Khasar et al., 1999). The results are shown in Fig. 2. The antibody to ϵ PKC recognizes only one band at 92 kDa in wild-type brain extracts and NG108/D2 whole-cell extracts. This band is absent in brain extracts from the ϵ PKC knockout mouse (Fig. 2), indicating that the ϵ PKC specific antibody is isozyme-specific. Similarly, the antibody to δ PKC recognizes only one band at 78 kDa in wild-type mouse brain extracts and in NG108/D2 whole-cell extracts (Fig. 2). This band is absent in brain extracts from the δ PKC knockout mouse, supporting the specificity of the δ PKC antibodies. In addition, no change was observed in localization of a Golgi marker after treatment with ethanol (data not shown), nor was there a change in cell morphology (Fig. 3B). Ethanol- and NPA-induced translocation of δ and ϵ PKC were not restricted to NG108–15/D2 cells. CHO cells expressing the D2 receptor (CHO/D2 cells) (Fishburn et al., 1995) also exhibited ethanol- and NPA-induced translocation of δ PKC to the nucleus and perinucleus and ϵ PKC to the cytoplasm (Fig. 1B). As for NG108–15/D2 cells, spiperone blocked dopamine-induced translocation of δ and ϵ PKC in CHO/D2 cells (Fig. 1B) but had no effect on ethanol-induced translocation (data not shown). NPA had no effect on δ and ϵ PKC localization in wild-type CHO cells that lack the D2R (data not shown). NPA-induced translocation of ϵ PKC as a function of time in CHO/D2 cells is illustrated in Fig. 4A; translocation is detectable at 5 min, maximal at 10 min. Translocation of ϵ PKC at 30 min is shown in Fig. 4B as a function of NPA concentration; half-maximal translocation occurs at $5 \pm 1 \times 10^{-9}$ M NPA. The decrease in ϵ PKC translocation at higher NPA concentrations is most likely caused by desensitization of the D2R. Qualitatively similar results were obtained with NG108–15/D2 cells (data not shown).

The Role of PLC in Ethanol and D2R Agonist-Induced Translocation of PKC. D2R activation increases phospholipase C (PLC) activity in several cell types (Vallar et al., 1990; Tang et al., 1994), producing diacylglycerol (DAG),

which in turn leads to activation and translocation of PKC (Mochly-Rosen, 1995). Ethanol also increases PLC and PKC activities in several cell types (Messing et al., 1991; DePetrillo and Liou, 1993; Kharbanda et al., 1993; Deitrich et al., 1996; Higashi et al., 1996; Mironov and Hermann, 1996). Because many PLC isozymes are activated by trimeric G proteins (Morris and Scarlata, 1997), we determined whether ethanol- and NPA-induced PKC translocation was inhibited by pertussis toxin (PTX), which inhibits activation of PLC by receptors coupled to $G\alpha_i/\alpha_o$. We found that PTX inhibited ethanol- and NPA-induced translocation of δ PKC and ϵ PKC (Fig. 5) in CHO/D2 cells. Similar results were obtained with NG108–15/D2 cells (data not shown). If PLC activation is required for ethanol-induced PKC translocation, then inhibition of PLC activity should prevent translocation. Indeed, we found that the PLC inhibitors U-73122 and Et-18-OCH_3 each inhibit ethanol-induced translocation of ϵ PKC in CHO/D2 cells (Table 1). Similar results were obtained for δ PKC in CHO/D2 cells and for δ and ϵ PKC in NG108–15/D2 cells (data not shown).

Synergy between Ethanol and D2R Activation. The data presented here suggest that ethanol and D2Rs share a common signaling pathway that results in activation and translocation of δ and ϵ PKC in a neural cell line and CHO cells expressing the D2R. Therefore, we expected that low concentrations of each agent might have either an additive or a synergistic effect on translocation of these PKC isozymes. Figure 6 shows the localization of δ (A) and ϵ (B) PKC in NG108/D2 cells that have been coincubated with or without 10^{-9} M NPA in the presence or absence of 5, 10, or 25 mM ethanol for 30 min. Neither NPA alone nor ethanol alone, when incubated with these cells for 30 min, causes translocation of ϵ PKC (Fig. 6B). However, when cells are coincubated with 10^{-9} M NPA and 10 or 25 mM EtOH for 30 min, translocation of ϵ PKC to the cytoplasm was observed (Fig. 6B). There was no synergy between 10^{-9} M NPA and 5 mM ethanol for translocation of ϵ PKC (Fig. 6B). Synergy of NPA and ethanol for translocation of δ PKC was different from that for ϵ PKC. Only coincubation with 10^{-9} M NPA and 25 mM ethanol caused δ PKC translocation from the Golgi area to the perinucleus and nucleus (Fig. 6A). There was no translocation of δ PKC at 5 and 10 mM ethanol in NG108/D2 cells whether incubated alone or with 10^{-9} M NPA. Therefore, ϵ

TABLE 1

ε PKC translocation in CHO/D2 cells

CHO/D2 cells were preincubated in the presence or absence of the PLC inhibitors ET-18-OCH_3 (10 μM) for 30 min or U73122 (1 μM) for 60 min and then further incubated for 30 min in the absence or presence of ethanol (200 mM) or NPA (50 nM).

	% Cells with Cytoplasmic Staining of ε PKC[a]	
Control	14 ± 3	$n = 6$
EtOH	93 ± 3*	$n = 6$
NPA	92 ± 6*	$n = 6$
EtOH + U73122	12 ± 4	$n = 3$
NPA + U73122	14 ± 6	$n = 3$
EtOH + ET-18-OCH_3	15 ± 3	$n = 3$
NPA + ET-18-OCH_3	14 ± 3	$n = 3$
U-73122	16 ± 4	$n = 3$
ET-18-OCH_3	14 ± 5	$n = 3$

[a] Percentage of cells with cytoplasmic staining for ε PKC calculated as described under *Experimental Procedures*.

* $p < 0.001$ compared with control cells in the absence or presence of the PLC inhibitors, student's t test.

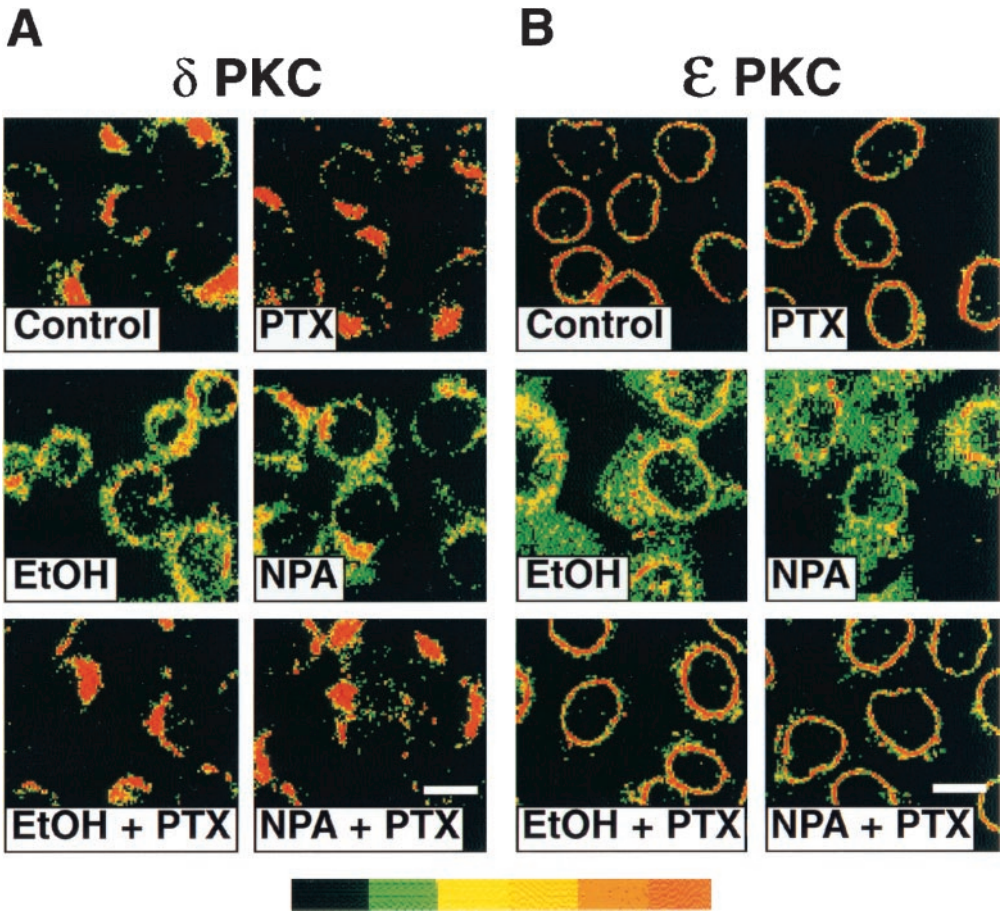

Fig. 5. Pertussis toxin prevents ethanol- and NPA-induced translation of δ (A) and ϵ (B) PKC. CHO/D2 cells were incubated overnight in the absence or presence of 50 ng/ml pertussis toxin (PTX) and then exposed to ethanol (200 mM) or NPA (50 nM) for 30 min. The data are representative of three experiments. (Scale bar, 10 μm; images are 600×)

PKC seems to be more sensitive to ethanol than δ PKC with respect to synergy.

Discussion

We show here that activation of the D2R expressed in NG108–15 (Fig. 1A) and CHO (Fig. 1B) cell lines causes translocation of δ and ϵ PKC to the perinucleus and cytoplasm, respectively. We have reported (Gordon et al., 1997) and confirmed here (Fig. 1) that ethanol also causes translocation of δ and ϵ PKC to similar sites in these cells. PTX (Fig. 5) and inhibitors of PLC (Table 1) inhibit both NPA and ethanol-induced translocation. Taken together, our results suggest that ethanol and NPA cause translocation of δ and ϵ PKC by activating PLC via a pertussis toxin-sensitive G protein, thus increasing DAG levels and causing activation and subsequent translocation of δ and ϵ PKC. Moreover, our data suggest that ethanol mimics D2R activation. The effects of ethanol and a dopamine agonist are synergistic because concentrations of ethanol as low as 25 mM and 10^{-9} M of the D2 agonist NPA, which alone do not cause translocation of ϵ PKC, together cause maximal translocation in NG108/D2 cells (Fig. 6B). δ PKC seems to be less sensitive to ethanol in the synergy experiments; 25 mM ethanol and 10^{-9} M NPA are required to cause translocation of δ PKC (Fig. 6A). Similar results were obtained at 25 mM ethanol for both PKC isozymes in CHO/D2 cells (data not shown), suggesting that

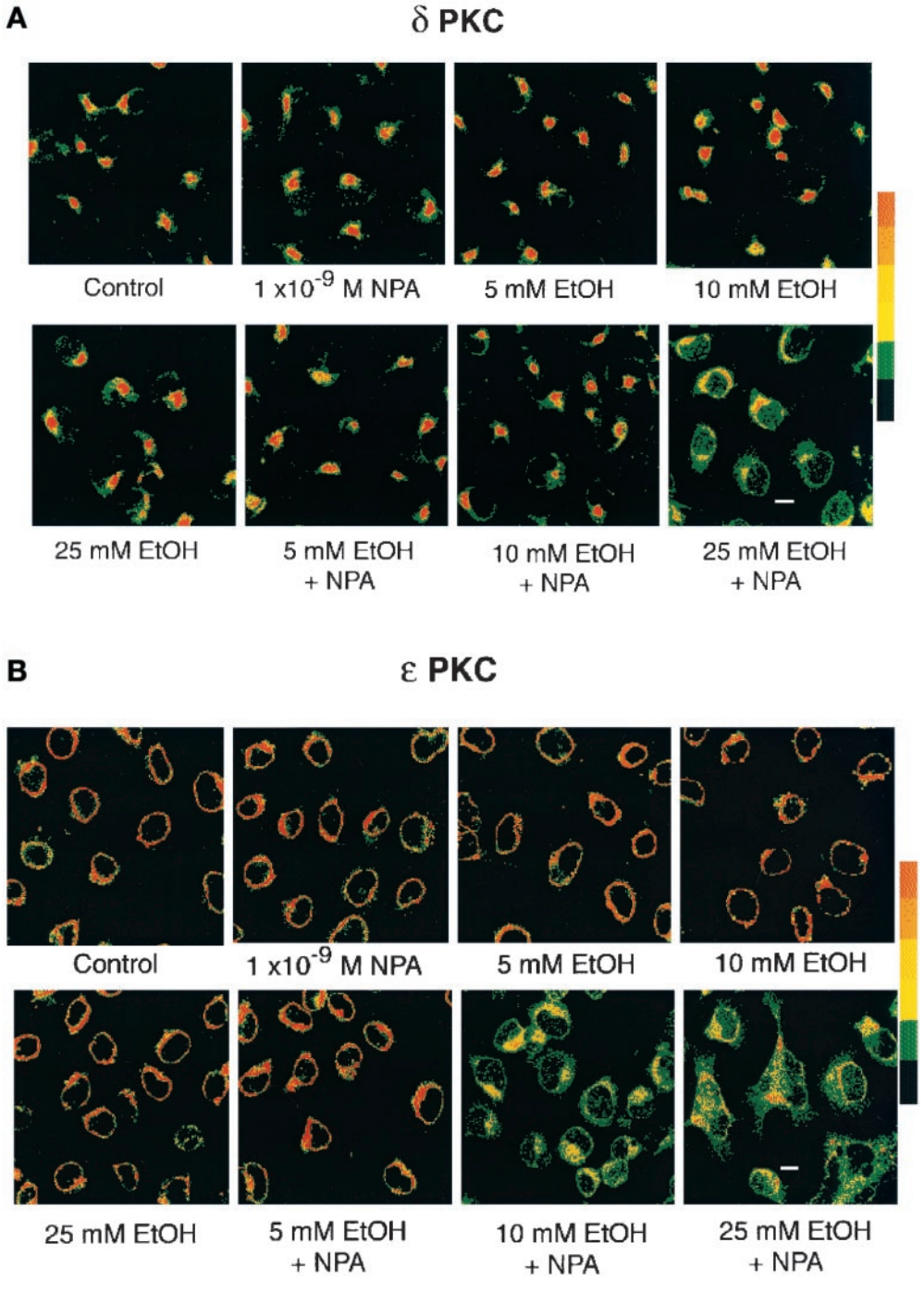

Fig. 6. Ethanol and NPA act synergistically to induce δ and ϵ PKC translocation. NG108–15/D2 cells were incubated at the indicated concentrations of ethanol, NPA, or ethanol plus NPA for 30 min, and the localization of δ PKC (A) and ϵ PKC (B) was determined. Control cells were incubated in defined media alone. The data are representative of three independent experiments. (Scale bar, 10 μm; images are 400×)

the synergy between NPA and ethanol is not cell-type specific.

Translocation of δ PKC from the Golgi area is primarily to the perinucleus and nucleus, whereas ϵ PKC translocates from the perinucleus to the cytoplasm (Fig. 1; Gordon et al., 1997). Although we cannot determine directly whether ethanol and NPA activate δ and ϵ PKC, our data and that of others suggest that these isozymes are activated. Ethanol activates PLC and PKC in many cell types (Messing et al., 1991; DePetrillo and Liou, 1993; Kharbanda et al., 1993; Deitrich et al., 1996; Higashi et al., 1996; Mironov and Hermann, 1996) and translocation of PKC isozymes has been shown to be sufficient for their activation (Mochly-Rosen, 1995; Mochly-Rosen and Gordon, 1998). Moreover, PLC inhibitors that prevent formation of DAG block δ and ϵ PKC translocation by ethanol or NPA (Table 1).

Mochly-Rosen and colleagues [see Mochly-Rosen (1995) and Mochly-Rosen and Gordon (1998) for review] have proposed that the site of localization of activated PKC isozymes is determined by the location of isozyme-specific receptors for activated PKC (RACKs). Since δ PKC is translocated to the perinucleus and nucleus by ethanol, NPA, and phorbol esters (Gordon et al., 1997), it seems likely that translocated δ PKC is active and its RACK is localized to the perinucleus and nucleus in NG108–15/D2 and CHO/D2 cells. δ PKC remains in the perinucleus and nucleus as long as ethanol is present, at least up to 4 days with 25 mM ethanol (Gordon et al., 1997). Therefore, it is possible that ethanol-induced nuclear δ PKC inappropriately regulates the expression of specific genes as long as ethanol is present. This may account for some ethanol-induced changes in gene expression [see Diamond and Gordon (1997) for review] that could regulate many cellular functions [see Olson et al. (1993) for review].

Ethanol and NPA also cause a striking translocation of ϵ PKC from the perinucleus to the cytoplasm in both NG108–15/D2 and CHO/D2 cells (Fig. 1). However, incubation of NG108–15 cells with phorbol esters causes translocation of ϵ PKC to the nucleus, not the cytoplasm (Gordon et al., 1997). These findings suggest that localization of ϵ RACK (β′COP; Csukai et al., 1997), may itself be regulated by intracellular signals.

Relevance to Alcoholism. Our results may provide insight into some cellular events that underlie behavioral responses to ethanol. Ethanol causes release of dopamine in the nucleus accumbens (Imperato and DiChiara, 1986; Weiss et al., 1992; McBride et al., 1993), which activates D2Rs shown to contribute to the behavioral effects of ethanol and other drugs of abuse (Hodge et al., 1996; Koob and Nestler, 1997). We show here that both ethanol and a D2 agonist cause δ and ϵ PKC translocation in cultured neural cells and CHO cells (Fig. 1) and that the effects of dopamine and ethanol are synergistic at low concentrations (Fig. 6). We propose, therefore, that dopamine neurotransmission via the D2R will be greatly enhanced by low concentrations of ethanol in vivo because maximal translocation of δ and ϵ PKC occurs at concentrations of ethanol and NPA together that are ineffective alone. Moreover, because ethanol also causes translocation of these kinases in cells lacking D2 receptors (Gordon et al., 1997), there may also be synergy with other neurotransmitters in different regions of the brain. We have previously shown that δ and ϵ PKC remain at the new sites in NG108–15 cells as long as ethanol is present (Gordon et al., 1997). Therefore, δ and ϵ PKC might remain at these new sites in neurons in vivo as long as ethanol is present, thereby limiting or enhancing the normal function of some neurotransmitter receptors.

In summary, we show here that ethanol mimics D2R-induced translocation of δ and ϵ PKC and that there is synergy between low concentrations of ethanol and a D2R agonist, enabling ethanol to amplify D2R responses. Synergy of ethanol- and D2R-regulated translocation of δ and ϵ PKC in D2R-enriched areas, such as the nucleus accumbens, may underlie some of the behaviors associated with alcoholism, particularly, craving and addiction.

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