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Ethanol enhances growth factor activation of mitogen-activated protein kinases by a protein kinase C-dependent mechanism

(nerve growth factor/fibroblast growth factor/PC12 cells)

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ABSTRACT **Excessive alcohol consumption alters neuronal growth and causes striking elongation of axons and dendrites in several brain regions. This could result from increased sensitivity to neurotrophic factors, since ethanol markedly enhances nerve growth factor (NGF)- and basic fibroblast growth factor (bFGF)-stimulated neurite outgrowth in the neural cell line PC12. The mechanism by which ethanol enhances growth factor responses was investigated by examining activation of mitogen-activated protein kinases (MAP kinases), a key event in growth factor signaling. Ethanol (100 mM) increased NGF- and bFGF-induced activation of MAP kinases. This increase, like ethanol-induced increases in neurite outgrowth, was prevented by down regulation of β, δ, and ε protein kinase C (PKC) isozymes. Since chronic ethanol exposure specifically upregulates δ and ε PKC, these findings suggest that ethanol promotes neurite growth by enhancing growth factor signal transduction through a δ or ε PKC-regulated pathway.**

Medical complications of alcohol abuse contribute to more than 20% of hospital admissions in the United States (1). Several neurological disorders are associated with alcoholism and many appear to result from a direct neurotoxic effect of alcohol (2). One mechanism by which alcohol injures the nervous system is by altering the growth of axons and dendrites (neurites). Although early studies showed that ethanol inhibited neurite growth in some neurons (3, 4), several reports (5–10) indicate that ethanol markedly enhances neurite growth in several brain regions. For example, in adult rats, chronic ethanol exposure increases the length of dendrites in cerebellar Purkinje cells and hippocampal dentate granule cells and the number of dendritic spines on dentate granule cells and somatosensory cortical neurons (5–8). Prenatal ethanol exposure increases dendritic arborization in layer Vb somatosensory corticospinal neurons and causes hyperdevelopment of hippocampal dentate granule cell axons (9, 10). Ethanol also enhances neurite outgrowth in cultured cerebellar neurons (11) and in PC12 cells treated with nerve growth factor (NGF) or basic fibroblast growth factor (bFGF) (12, 13). Increases in neurite length could disturb neuronal function by delaying nerve conduction and by interfering with remodeling of neurites and synapses during development and learning.

We have used PC12 cells as a model system to study mechanisms by which ethanol alters neurite outgrowth. We previously reported that enhancement of NGF-induced neurite outgrowth by ethanol was inhibited by downregulation of β, δ, and ε isoforms of protein kinase C (PKC) (14). We also found that exposure to 25–100 mM ethanol for 2–6 days increased levels of mRNA (15) and protein (16) for δ and ε PKC and activated PKC in PC12 cells (16). Taken together, these findings suggest that ethanol enhances neurite formation by activating specific isozymes of PKC.

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One mechanism by which ethanol and PKC could modulate NGF-induced neurite outgrowth is by altering NGF signal transduction. A key event in growth factor signaling is activation of the closely related protein-serine/threonine kinases known as mitogen-activated protein kinases (MAP kinases; also called extracellular signal-regulated kinases, or ERKs) $p44^{mapk}$ (ERK-1) and $p42^{mapk}$ (ERK-2) by phosphorylation on tyrosine and threonine residues (17, 18). MAP kinases phosphorylate a variety of substrates, including the nuclear transcription factors c-Myc (19) and c-Jun (20), $pp90^{rsk}$ (21), microtubule-associated protein 2 (22), tyrosine hydroxylase (23), and phospholipase A_2 (24, 25). Thus, MAP kinases mediate several actions of growth factors on translation, transcription, cytoskeletal function, and cell signaling. In many cell types (26), including PC12 cells (27, 28), tumor-promoting phorbol esters that activate PKC stimulate phosphorylation and activation of MAP kinases. Since ethanol activates PKC (16), we investigated whether ethanol enhances neurite outgrowth in PC12 cells by stimulating phosphorylation and activation of MAP kinases through a PKC-dependent mechanism.

MATERIALS AND METHODS

Materials. NGF (2.5 S) was a gift from William Mobley (University of California, San Francisco). Phorbol 12-myristate 13-acetate (PMA) was obtained from LC Services (Woburn, MA). The nonapeptide APRTPGGRR was synthesized by the University of California, San Francisco, Biomolecular Resource Center. Mouse monoclonal anti-phosphotyrosine antibody (4G10), mouse monoclonal antibody to ERK-2 (erk2), and rabbit polyclonal antibody to ERK-1 (erk1-CT) were from Upstate Biotechnology (Lake Placid, NY). Mouse monoclonal antibody against $p34^{cdc2}$ (PSTAIRE) was a gift from Steven L. Pelech (Kinetek Biotechnology, Vancouver, BC). Antibodies against α, δ, ε, and ζ PKC were from Life Technologies (Gaithersburg, MD). Anti-β (I and II) PKC antibody was from Seikagaku America (Rockville, MD), and antibodies to β_I, β_{II}, and γ PKC were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody to η PKC was a gift from David Burns of Sphinx Pharmaceuticals (Durham, NC). Goat anti-mouse and goat anti-rabbit peroxidase-conjugated antibodies were from Boehringer Mannheim. Enhanced chemiluminescence (ECL) detection reagents, ^{125}I-labeled NGF, and [γ-^{32}P]ATP were from Amersham.

Cell Culture. PC12 cells were maintained at 37°C in a humidified atmosphere of 10% CO_2/90% air in Dulbecco's modified Eagle's medium with 10% horse serum, 5% fetal

Abbreviations: PKC, protein kinase C; NGF, nerve growth factor; bFGF, basic fibroblast growth factor; MAP kinase, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; PMA, phorbol 12-myristate 13-acetate.

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bovine serum, 2 mM glutamine, penicillin (50 units/ml), and streptomycin (50 μg/ml). Cells were grown in 75-cm^2 flasks with or without 100 mM ethanol and were replated on polyornithine-treated tissue culture dishes before carrying out experiments. To prevent evaporation of ethanol, flasks were tightly capped, dishes were wrapped in Parafilm, and medium was changed daily, as described (29).

Immunoblotting. PC12 cells were grown in flasks for 4 days with or without 100 mM ethanol and then were plated on polyornithine-coated 100-mm dishes at 6–8 × 10^6 cells per dish in the continued presence or absence of ethanol. The next day 100 nM or 1 μM PMA was added to some dishes for 24 hr to downregulate PKC isozymes. Dishes were then rinsed with buffer A (120 mM NaCl/1.4 mM $CaCl_2$/0.8 mM $MgSO_4$/1 mM NaH_2PO_4/10 mM glucose/25 mM Hepes, pH 7.4). NGF (50 ng/ml) was added in buffer A with or without 10 nM PMA and cells were incubated at 37°C for 2.5–60 min. Cells were then washed twice in ice-cold phosphate-buffered saline and scraped off plates in lysis buffer [10 mM sodium phosphate, pH 6.8/0.1 mM sodium pyrophosphate/0.4% SDS/1 mM Na_3VO_4/1 mM phenylmethylsulfonyl fluoride with leupeptin (25 μg/ml) and soybean trypsin inhibitor (25 μg/ml)]. Lysates were heated at 100°C for 10 min and samples were taken for protein determination. Concentrated sample buffer was added to yield a final solution containing 62.5 mM Tris·HCl (pH 6.8), 2% SDS, 10% glycerol, and 5% 2-mercaptoethanol, and 90-μg samples were electrophoresed in 11% polyacrylamide gels. Proteins were electrophoretically transferred to nitrocellulose membranes (Hybond-C extra, Amersham), which were then blocked for 1 hr at 25°C with 2% bovine serum albumin in Tris-buffered saline (20 mM Tris·HCl, pH 7.4/137 mM NaCl) containing 0.2% Tween 20 (TBS-T). Blots were then incubated with anti-phosphotyrosine antibody (1 μg/ml in blocking solution) for 2–3 hr at 25°C and washed for 10 min three times in TBS-T. After incubation for 1 hr at 25°C with peroxidase-conjugated anti-mouse antibody (1:1000 dilution), blots were washed four times in TBS-T, incubated with ECL detection reagent, and exposed to Kodak X-Omat AR film. Autoradiograms were analyzed by scanning densitometry and values were normalized against Coomassie blue-stained gels as described (16). For reprobing, blots were incubated in stripping buffer (62.5 mM Tris, pH 6.8/2% SDS/100 mM 2-mercaptoethanol) for 30 min at 70°C and washed for 10 min three times in TBS-T. Blots were then immunostained as described above with antibody to ERK-1 or ERK-2 (2 μg/ml). For detection of ERK-1 immunoreactivity, peroxidase-conjugated goat anti-rabbit IgG (1:1000 dilution) was used as the secondary antibody. Immunoblot analysis of PKC isozymes was carried out as described (16). Analysis of p34^{cdc2} immunoreactivity was performed with PSTAIRE antibody (1:500 dilution) (30).

MAP Kinase Assay. Cells were incubated in buffer A with NGF or PMA as described above for immunoblotting. After three rinses in ice-cold phosphate-buffered saline, cells were incubated at 4°C for 25 min in 20 mM Tris·HCl, pH 7.4/50 mM β-glycerophosphate/1% (vol/vol) Triton-X-100/1 mM Na_3VO_4/1 mM phenylmethylsulfonyl fluoride/5 mM dithiothreitol with leupeptin (20 μg/ml) and aprotinin (20 μg/ml). Lysates were cleared by centrifugation at 12,000 × *g* for 10 min, and supernatants were diluted to a protein concentration of 1.25 mg/ml. The reaction was started by adding 20 μl of lysate to 20 μl of reaction buffer containing 20 mM $MgCl_2$, 20 mM Hepes (pH 7.4), 1.4 nmol of [γ-^{32}P]ATP (7 × 10^5 dpm/nmol), and 40 μg of APRTPGGRR (31). The reaction was stopped after incubation for 8 min at 25°C by adding 20 μl of 0.2 M ATP/0.2 M EDTA. Samples (50 μl) were spotted onto phosphocellulose papers, which were washed three times in 1% phosphoric acid and once in 75 mM sodium phosphate (pH 7.5). Kinase activity was calculated as the difference between radioactivity in the presence and absence of APRTPGGRR. Under these conditions, phosphorylation of APRTPGGRR increased linearly for at least 10 min.

Phenyl-Superose Chromatography. PC12 cells were grown in 75-cm^2 flasks for 6 days in medium containing 100 mM ethanol. NGF (50 ng/ml) was then added for 20 min at 37°C. Flasks were rinsed three times with ice-cold buffer A and cells were lysed in 1.5 ml of buffer B [20 mM Mops, pH 7.2/60 mM β-glycerophosphate/5 mM EGTA/1 mM EDTA/1 mM Na_3VO_4/1 mM dithiothreitol/2 mM phenylmethylsulfonyl fluoride with leupeptin (20 μg/ml) and aprotinin (20 μg/ml)]. After incubation for 30 min at 4°C, cell debris was removed by centrifugation at 12,000 × *g* for 10 min. NaCl was added to 500 mM and proteins were separated by FPLC. Samples containing 1.5 mg of protein were loaded at 0.2 ml/min onto a phenyl-Superose FPLC column (Pharmacia) equilibrated with buffer C (13.3 mM Mops, pH 7.2/40 mM β-glycerophosphate/3.3 mM EGTA/0.67 mM Na_3VO_4/0.03 mM NaF/0.67 mM dithiothreitol/500 mM NaCl). After the column was washed with 5 ml of buffer C, proteins were eluted with a 15-ml linear 0–60% ethylene glycol, 500–25 mM NaCl gradient in buffer A at 0.2 ml/min, and 0.5-ml fractions were collected. Samples (100 μl) were immediately assayed for phosphotyrosine, ERK-1, ERK-2, and p34^{cdc2} immunoreactivity. MAP kinase activity in each fraction was assayed in 60-μl samples with reagents and conditions described above for measuring MAP kinase activity in cell lysates, except that the reaction volume was 80 μl and contained 0.5 mM dithiothreitol and 30 mM β-glycerophosphate.

Miscellaneous Procedures. Protein concentrations were determined by the Bradford method (32) with bovine IgG standards. Data were analyzed by ANOVA and where $P < 0.05$, the significance of differences between means was evaluated by the Newman–Keuls post-hoc test.

RESULTS

Ethanol Increases MAP Kinase Tyrosine Phosphorylation. NGF stimulated tyrosine phosphorylation of 44- and 42-kDa proteins (Fig. 1 *A* and *C*) which correspond to ERK-1 and ERK-2 (33). Phosphorylation was maximal after 5 min (peak phase) and then persisted at a lower level (plateau phase) for at least 1 hr (Fig. 1 *A* and *C*). Pretreatment of cells with 100 mM ethanol for 6 days markedly increased NGF-stimulated phosphotyrosine immunoreactivity (Fig. 1 *A* and *C*). This was due to increased phosphorylation and not to increased abundance of protein, since the amount of ERK-1, as determined by Western analysis, was not increased by ethanol (Fig. 1*A*). Although in some experiments ethanol slightly enhanced the peak phase of phosphorylation (Fig. 1 *A* and *C*), this was not apparent in all experiments. However, ethanol consistently increased the plateau phase of MAP kinase phosphorylation (Fig. 1 *A* and *C*), maintaining it near levels achieved during the peak phase. Ethanol also increased the plateau phase of bFGF-induced MAP kinase phosphorylation (Fig. 1*B*). Ethanol alone did not stimulate tyrosine phosphorylation of these MAP kinases (Fig. 1 *A* and *B*, 0 min).

Enhanced Phosphorylation of MAP Kinases by Ethanol Is PKC-Dependent. One mechanism by which ethanol could promote MAP kinase phosphorylation is by activating PKC. High concentrations (1 μM) of phorbol esters such as PMA, which fully activate several PKC isozymes (34), modestly stimulate MAP kinase in PC12 cells (27, 28). Prolonged exposure to ethanol increases levels of δ and ε PKC and increases PKC-mediated phosphorylation in PC12 cells (16). Since PKC regulates MAP kinases and is activated by ethanol, we investigated whether PKC mediates ethanol's enhancement of MAP kinase phosphorylation.

We first examined whether activation of PKC by phorbol esters mimics the effect of ethanol and enhances MAP kinase phosphorylation stimulated by NGF or bFGF. For these studies we

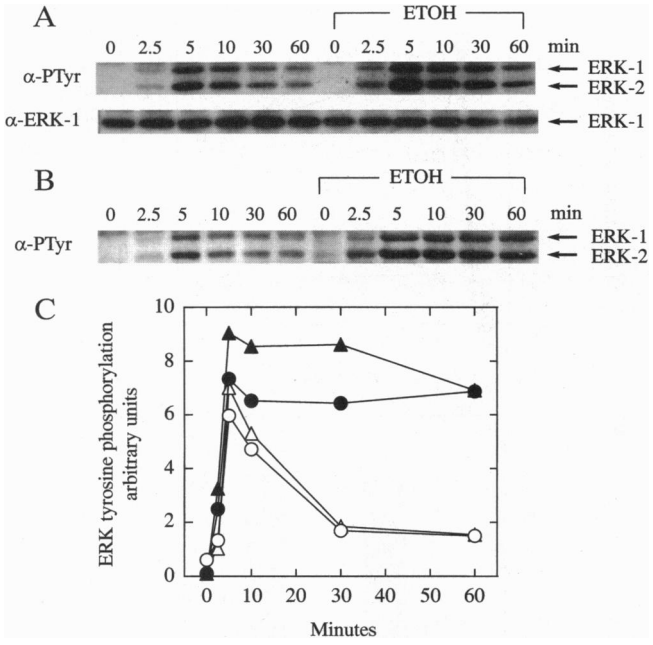

FIG. 1. Ethanol-enhanced tyrosine phosphorylation of MAP kinases. (*A*) Cells cultured with (ETOH) or without 100 mM ethanol for 6 days were exposed to NGF (50 ng/ml) for 0–60 min, as indicated. Lysates were analyzed by Western blotting for phosphotyrosine immunoreactivity (α-PTyr). The blot was also stripped and probed for ERK-1 protein (α-ERK-1). (*B*) Cells cultured with (ETOH) or without 100 mM ethanol for 6 days were exposed to bFGF (50 ng/ml) for 0–60 min and lysates were analyzed by Western blotting with anti-phosphotyrosine antibody. Scanning densitometry showed that after 5 min, there was no significant difference in tyrosine phosphorylation of ERK-1 or ERK-2 in ethanol-treated and control cells. However, after 30 min, ERK-1 phosphorylation was increased 2.5-fold and ERK-2 phosphorylation was increased 4.8-fold in ethanol-treated cells compared with control cells. (*C*) ERK-1 and ERK-2 immunoreactive bands detected with anti-phosphotyrosine antibody in *A* were quantified by scanning densitometry. Values were normalized against Coomassie blue-stained gels to correct for protein loading, as described (16).

used PMA at a concentration (10 nM) that submaximally activates PKC (35) to mimic modest activation of PKC by ethanol (16). Like ethanol, 10 nM PMA alone did not stimulate MAP kinase phosphorylation (Fig. 2*B*) but markedly enhanced phosphorylation induced by NGF or bFGF (Fig. 2*A*).

We next examined whether PKC is required for ethanol's enhancement of MAP kinase phosphorylation. PC12 cells can be depleted of several PKC isozymes by treatment with high concentrations of phorbol esters for several hours (14, 36). Using isozyme-specific antibodies, we found that our PC12 clone contained α, β_{II}, δ, ε, and ζ PKC, but not β_I, γ, or η PKC (Fig. 3*A* and unpublished observations). Pretreatment with 100 nM PMA for 24 hr downregulated β, δ, and ε PKC (Fig. 3*A*). α PKC was downregulated only after treatment with 1 μM PMA (Fig. 3*A*) whereas ζ PKC was resistant to downregulation at all PMA concentrations tested (data not shown). Similar results were observed in cells cultured in 100 mM ethanol for 5 days before addition of 100 nM PMA for 24 hr (Fig. 3*A*). Pretreatment with 100 nM PMA for 24 hr reduced NGF-induced MAP kinase phosphorylation and prevented enhancement by ethanol (Fig. 3 *B* and *C*). No further reduction in MAP kinase phosphorylation was observed in cells pretreated for 24 hr with 1 μM PMA (data not shown).

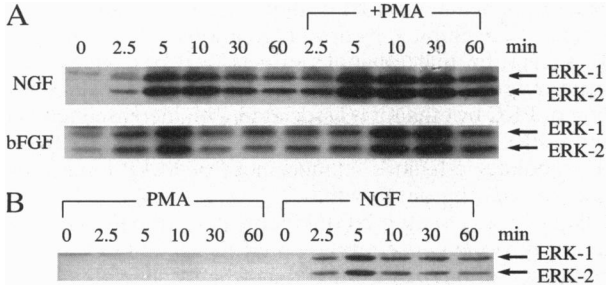

FIG. 2. PMA-enhanced tyrosine phosphorylation of MAP kinases. (*A*) MAP kinase phosphorylation in cells treated with NGF or bFGF with or without 10 nM PMA. (*B*) MAP kinase phosphorylation after treatment with 10 nM PMA alone or NGF (50 ng/ml) alone.

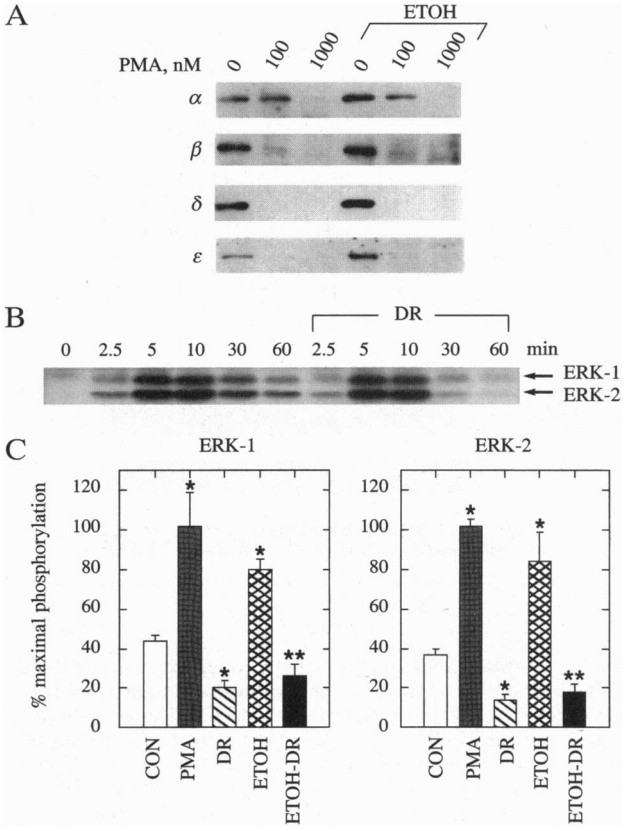

FIG. 3. PKC downregulation and ERK phosphorylation. (*A*) Western blots of PKC isozymes in homogenates of cells cultured with (ETOH) or without 100 mM ethanol for 6 days and exposed to 100 or 1000 nM PMA during the last 24 hr. (*B*) Western blot of ERK-1 and ERK-2 tyrosine phosphorylation in cells pretreated with 100 nM PMA for 24 hr [to downregulate (DR) PKC isozymes] and then exposed to NGF (50 ng/ml) for 0–60 min. (*C*) ERK tyrosine phosphorylation after treatment with NGF (50 ng/ml) for 20 min. Treatment groups: NGF alone (CON); NGF plus 10 nM PMA (PMA); 100 nM PMA for 24 hr to downregulate PKC isozymes before addition of NGF (DR); 100 mM ethanol for 6 days followed by addition of NGF for 20 min (ETOH); 100 mM ethanol for 5 days and ethanol plus 100 nM PMA for another 24 hr before addition of NGF (ETOH-DR). Data are expressed as a percentage of maximal phosphorylation measured in parallel samples treated with NGF alone for 5 min (peak phase) and are mean ± SE values from 4–12 experiments. *, Significantly different from control; **, significantly different from ETOH and from CON, but not from DR (ANOVA, Newman–Keuls test; $\alpha = 0.05$).

Ethanol Increases NGF-Stimulated MAP Kinase Activity. To examine whether PMA- and ethanol-induced changes in MAP kinase phosphorylation lead to corresponding increases in MAP kinase activity, we measured MAP kinase activity in cell lysates. MAP kinase activity can be assayed by measuring the phosphorylation of known MAP kinase substrates such as myelin basic protein or microtubule-associated protein 2. However, these substrates are phosphorylated by several other kinases, making it difficult to use them to selectively detect MAP kinase activity in cell homogenates. APRTPGGRR is a peptide derived from sequences surrounding the MAP kinase phosphorylation

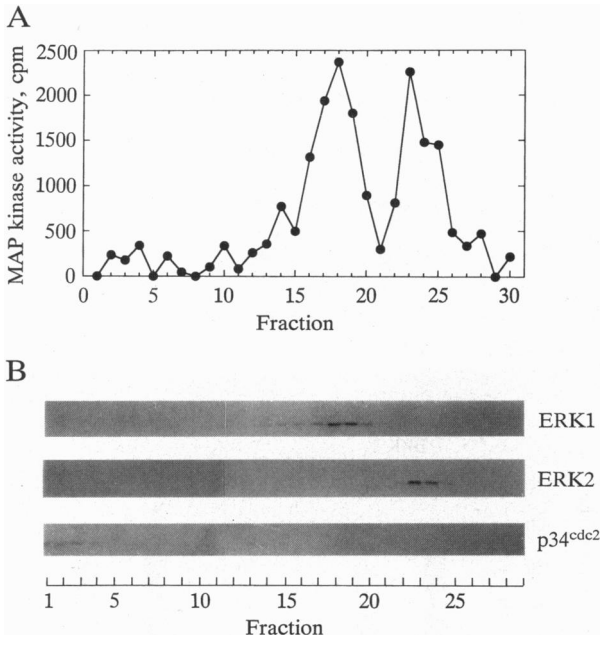

FIG. 4. APRTPGGRR kinase activity comigrates with ERK-1 and ERK-2, but not with $p34^{cdc2}$, on a phenyl-Superose column. PC12 cells were exposed to 100 mM ethanol for 6 days and then to NGF (50 ng/ml) for 20 min. Cell lysates were separated over a phenyl-Superose column as described in *Materials and Methods*. (*A*) MAP kinase activity in each fraction. (*B*) Western blots of samples from each fraction probed with antibodies to ERK-1, ERK-2, and $p34^{cdc2}$.

site of myelin basic protein (Thr-97) and is selectively phosphorylated by proline-directed kinases such as MAP kinases (31). We used this peptide to develop an assay for MAP kinase activity in crude PC12 cell lysates. After phenyl-Superose chromatography of cell lysates, kinase activity against this peptide was detected as two peaks (Fig. 4*A*) that were coeluted with ERK-1 and ERK-2 immunoreactivity (Fig. 4*B*). In contrast, no activity was detected in fractions containing $p34^{cdc2}$, another proline-directed kinase. Thus, we used APRTPGGRR to assay MAP kinase activity in PC12 cell lysates.

NGF stimulated MAP kinase activity with a biphasic time course (Fig. 5*A*, CON) which correlated well with the time course for stimulation of MAP kinase phosphorylation (Fig. 1 *A* and *C*). Pretreatment of cells with 100 mM ethanol for 5–6 days markedly enhanced the plateau phase of NGF-stimulated MAP kinase activity (Fig. 5, ETOH). Similar enhancement occurred in cells treated simultaneously with NGF and 10 nM PMA (Fig. 5, PMA). The effects of ethanol pretreatment and 10 nM PMA were not additive (Fig. 5*B*, ETOH+PMA), suggesting that these treatments act by a common mechanism. Downregulation of β, δ, and ε PKC reduced the plateau phase of NGF-stimulated activity and blocked enhancement by ethanol (Fig. 5, DR and ETOH-DR). Ethanol alone did not stimulate MAP kinase activity, and acute addition of ethanol only during the time of exposure to NGF did not alter NGF-induced activation of MAP kinase (data not shown).

DISCUSSION

The major finding of this paper is that chronic exposure to ethanol enhances NGF-stimulated phosphorylation and activation of MAP kinases. In PC12 cells, MAP kinases appear to be important for neurite formation, since purine analogs that inhibit MAP kinases prevent neurite outgrowth induced by NGF (37). The second, plateau phase of MAP kinase activation by NGF appears particularly important since NGF and bFGF, which induce neurite outgrowth in PC12 cells, cause prolonged MAP kinase phosphorylation, whereas epidermal

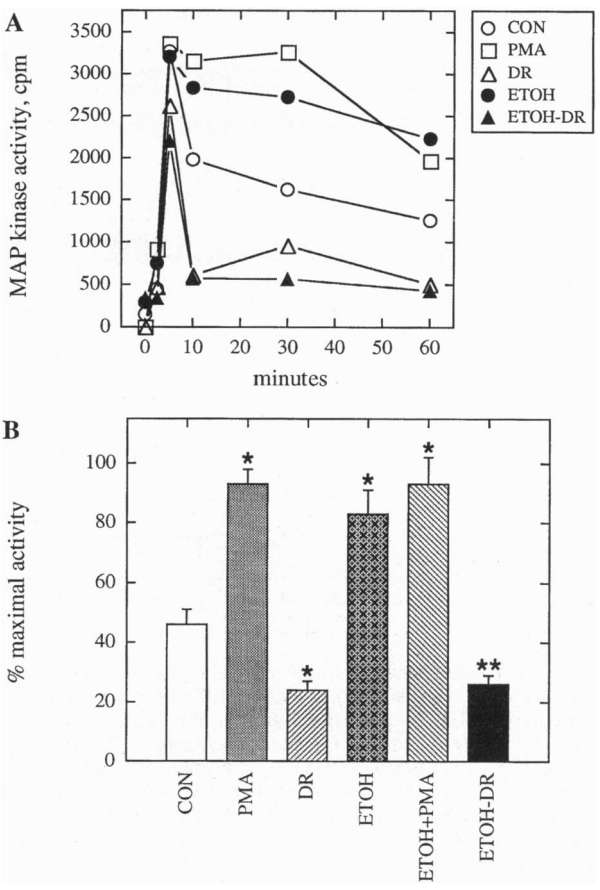

FIG. 5. Regulation of MAP kinase activity by ethanol and PMA. (*A*) Time course of MAP kinase activation after treatment with NGF (50 ng/ml) and ethanol or PMA. (*B*) MAP kinase activity after treatment with NGF for 20 min. Treatment groups are the same as those described in Fig. 3*C*. In addition, some cultures were exposed to 100 mM ethanol for 6 days before treatment with NGF plus 10 nM PMA for 20 min (ETOH+PMA). Values for MAP kinase activity in *B* are expressed as a percentage of maximal activity measured in parallel samples treated with NGF alone for 5 min and are mean ± SE values from 4–10 experiments. *, Significantly different from control; **, significantly different from ETOH and from CON, but not from DR (ANOVA, Newman–Keuls test, $\alpha = 0.05$).

growth factor, which does not induce neurites in PC12 cells, causes only transient MAP kinase phosphorylation (38). Therefore, enhancement of the plateau phase of MAP kinase activation probably underlies ethanol's ability to increase NGF- and bFGF-induced neurite outgrowth.

The other important finding of this study is that ethanol regulates activation of MAP kinases by a PKC-dependent mechanism. Ethanol's enhancement of NGF-induced MAP kinase activation was prevented by downregulation of β, δ, and ε PKC. Enhancement of NGF-induced neurite outgrowth by ethanol is also inhibited by downregulation of β, δ, and ε PKC (14), suggesting that ethanol's effects on MAP kinases and neurite outgrowth require similar PKC isozymes. Since activation of PKC by ethanol is associated with increased levels of δ and ε PKC (16), it is likely that δ or ε PKC, rather than β PKC, mediates ethanol's enhancement of MAP kinase activation and neurite outgrowth.

Ethanol does not alter NGF binding (14), or NGF and FGF receptor tyrosine phosphorylation (R.R. and R.O.M., unpublished observations), indicating that ethanol regulates growth factor signal transduction at a site distal to NGF or FGF receptors. Studies with phorbol esters indicate at least two possible sites at which ethanol could act. First, ethanol could increase activation of the GTPase Ras. Ras is activated by

NGF and is required for activation of MAP kinases by NGF (28). Ras is active when GTP is bound and is inactivated by the GTPase-activating protein Ras-GAP, which promotes the formation of inactive Ras·GDP (39). In lymphocytes, activation of PKC inhibits Ras-GAP and increases the formation of Ras·GTP (40). Therefore, ethanol could enhance MAP kinase activation by inhibiting Ras-GAP via PKC. Second, ethanol may promote PKC-mediated activation of the serine/threonine kinase Raf-1. NGF activates Raf-1 by a Ras-dependent pathway (41), and activated Raf-1 in turn phosphorylates and activates the dual-specificity tyrosine/threonine kinases that activate MAP kinases (42, 43). Recombinant α, β, and γ PKC expressed in insect cells (44) and α PKC in fibroblasts (45) activate Raf-1, and studies in fibroblasts indicate that α PKC activates Raf-1 by direct phosphorylation (45). Thus ethanol may stimulate phosphorylation of Raf-1 by PKC. This may act synergistically with NGF-induced activation of Raf-1 via Ras to increase Raf-1 activity and subsequent MAP kinase activation.

We found that the initial phase of NGF-stimulated MAP kinase phosphorylation and activation was not altered by PMA or ethanol. Instead, ethanol or 10 nM PMA prevented the subsequent decline in MAP kinase phosphorylation and activity. Moreover, this decline in MAP kinase phosphorylation and activity was accentuated by downregulation of PKC. This suggests that PKC may negatively regulate phosphatases that dephosphorylate and deactivate MAP kinases. Recently, 3CH134 and PAC-1 have been identified as the first members of what appears to be a family of dual-specificity threonine/tyrosine phosphatases that are induced by mitogens and deactivate MAP kinases (46–48). Inhibition of the expression or function of these phosphatases might be an important mechanism by which ethanol and activators of PKC sustain MAP kinase activity.

Our results support our previous finding that specific PKC isozymes mediate enhancement of neurite outgrowth by ethanol (14). This may cause injury to the nervous system by disturbing neural development and plasticity. We are hopeful that identification of specific PKC isozymes that mediate ethanol's effects and the development of PKC isozyme-specific antagonists will lead to new treatments for some alcohol-related neurologic disorders.

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