Biochemical Pharmacology, Vol. 51, pp. 661–668, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
SSDI 0006-2952(95)02239-2

Potentiation of Mitogen-Activated Protein Kinase by Ethanol in Embryonic Liver Cells*

Marpadga A. Reddy and Shivendra D. Shukla†

DEPARTMENT OF PHARMACOLOGY, UNIVERSITY OF MISSOURI SCHOOL OF MEDICINE, COLUMBIA, MO 65212, U.S.A.

ABSTRACT. Ethanol modulates agonist responses in liver cells, which are the major site of ethanol metabolism. Mitogen-activated protein kinases (MAPKs) are involved in the integration of multiple signaling pathways leading to cellular responses. However, the effect of ethanol on liver MAPK is not known. To this end, we studied the activation of MAPK in a normal mouse embryonic liver cell line (BNLCL2) after acute and chronic exposure to ethanol. Acute exposure to ethanol (0–400 mM) for 1 hr had no effect on either basal or serum- and phorbol-12-myristate-13-acetate (PMA)-stimulated MAPK activity. Chronic exposure to ethanol (0–400 mM) for 24 hr potentiated the stimulation of MAPK by serum, PMA, or thrombin. Maximum potentiation was observed with 200 mM ethanol (2- to 3-fold higher than control cells). Chronic exposure had no significant effect on epidermal growth factor-stimulated MAPK activity. In-gel MAPK assay of cytosolic extracts and of immunoprecipitates obtained with MAPK antibody demonstrated that ethanol potentiated the activation of both p42 and p44 MAPKs. When cells were pretreated with pertussis toxin, the potentiation by ethanol was abolished. It is concluded that ethanol potentiates MAPK in fetal liver cells by a pertussis toxin-sensitive G-protein-dependent mechanism. BIOCHEM PHARMACOL 51;5:661–668, 1996.

KEY WORDS. MAP kinases; PKC; protein tyrosine kinases; G-proteins; mouse embryonic liver; ethanol

Alcohol abuse is associated with deleterious effects on several organs of the body, particularly the liver and brain. The liver is exposed intermittently to high doses of ethanol since it is the major site of ethanol metabolism. Exposure to ethanol leads to both short- and long-term changes in liver function. Ethanol alters hepatic carbohydrate and lipid metabolism, as well as protein and DNA synthesis, which leads to liver dysfunction and cirrhosis [1, 2]. Ethanol inhibits the proliferation of hepatocyte cultures [3] and interferes with liver regeneration following partial hepatectomy or chemical injury [4–7]. The effects of ethanol have been attributed to changes in various signal transduction processes in liver cells. Signal transduction components modulated by ethanol are: phospholipase C [8], adenylate cyclase [9], protein tyrosine kinases [10–13] and PKC‡ [14–16]. In liver, acute treatment of ethanol activates PLC and mobilizes calcium [17], and chronic exposure desensitizes the hepatocytes to hormonal stimuli [18]. Ethanol also inhibits cyclic AMP-dependent signaling in regenerating rat liver [19]. Changes in the function and expression of G-proteins and PKC have been implicated in ethanol-mediated responses [8, 9, 19–23].

Liver regeneration is a complex process and requires multiple signaling pathways and activity of both protein tyrosine kinases and serine/threonine kinases [24]. Recently, a group of serine/threonine kinases termed MAPK have emerged as key enzymes involved in the integration of signal transduction processes leading to various cell responses including proliferation and differentiation [25–28]. At least two isoforms of MAPKs, p42 and p44, are activated by several agents including receptor protein tyrosine kinases, G-protein-coupled receptors and phorbol esters. MAPKs are activated by phosphorylation on both tyrosine and threonine residues [29]. Multiple pathways activating MAPKs have been identified. Receptor protein tyrosine kinases activate ras proteins via the adapter proteins Grb2 and Sos [30, 31]. This leads to activation of c-raf, which activates MAPK kinase (MEK) [32, 33]. MEK, in turn, activates MAPKs by simultaneous phosphorylation on both tyrosine and threonine residues [34]. Other pathways involve seven transmembrane receptors coupled to G-proteins G_i and G_q [35, 36]. Activated α_i, α_q and $\beta\gamma$-complexes activate PKC through interaction with PLCβ. The PKC activates raf or MEK kinase leading to activation of MAPK [37–39]. Activated G_i and $\beta\gamma$-complexes can also activate MAPK pathways through ras proteins independent of PKC [40, 41]. The activated MAPKs, in turn, regulate key intracellular enzymes and transcription factors involved in many cellular processes [42]. Whether ethanol affects MAPKs is not known. Results of such an investigation are presented here and offer a unique

* Part of this work was presented at the Research Society on Alcoholism meeting held in Maui, Hawaii, June 1994. [Reddy MA and Shukla SD, Ethanol potentiates MAP kinase activation in fetal liver cells. **Alcohol Clin Exp Res 18:** 515, 1994].

† Corresponding author. Tel. (314) 882-2740; FAX (314) 884-4558.

‡ *Abbreviations:* PKC, protein kinase C; MAPK, mitogen-activated protein kinase; PMA, phorbol-12-myristate-13-acetate; MBP, myelin basic protein; FBS, fetal bovine serum; DMEM, Dulbecco's Modified Eagle's Medium; EGF, epidermal growth factor; PTX, pertussis toxin; and PLC, phospholipase C.

Received 25 April 1995; accepted 27 September 1995.

action of ethanol in the modulation of MAPK activity in normal fetal liver cells.

MATERIALS AND METHODS

Reagents

MBP from rabbit brain, protein kinase inhibitor peptide, calmidazolium, staurosporine, thrombin, PMA, EGF, β-glycerophosphate, and benzamidine were obtained from the Sigma Chemical Co. (St. Louis, MO). The protease inhibitors (aprotinin and leupeptin) and protein A Sepharose were from Boehringer–Mannheim (Indianapolis, IN). The MAPK antibody (Z033) and rabbit anti-mouse IgG were purchased from Zymed Laboratories Inc. (San Francisco, CA). [γ-^{32}P]ATP (3000 Ci/mmol) was from New England Nuclear (Boston, MA). Fetal bovine serum was purchased from GIBCO BRL (Gaithersburg, MD).

Cell Culture

Mouse embryonic liver cells (BNLCL2), which are normal and non-transformed cells, were grown in DMEM high glucose containing 10% FBS, penicillin (100 U/mL), streptomycin (100 μg/mL) and L-glutamine (2 mM) at 37° under 5% CO_2. The cells (80% confluent) were starved in DMEM containing 0.1% FBS in the presence or absence of ethanol for 24 hr prior to stimulation with agonists. The cells grown in dishes were wrapped with parafilm to prevent evaporation of ethanol. In the initial experiments, ethanol-treated cells and control cells were incubated in separate incubators to prevent the effects of ethanol vapors on untreated (control) cells. Later, it was observed that there was no difference between the results obtained with cells incubated in the same or separate incubators.

Preparation of Cellular Extracts

The cells were washed twice with PBS (136 mM sodium chloride, 2.68 mM potassium chloride, 10 mM Na_2HPO_4, and 1.76 mM KH_2PO_4, pH 7.4), and stimulated with the appropriate agent for 5 min in DMEM containing 0.1% FBS at 37°. Cells were cooled immediately by placing the dishes onto ice and rinsed twice with ice-cold PBS followed by one rinse with ice-cold extraction buffer [50 mM β-glycerophosphate, pH 7.3, 1.5 mM EGTA, 0.1 mM Na_3VO_4, 1 mM dithiothreitol, 1 mM benzamidine, leupeptin (10 μg/mL), and aprotinin (10 μg/mL)]. Cells were scraped into extraction buffer and sonicated for 5 sec using a microtip probe at a power setting of 5 in a Vibracell Ultrasonic processor (model VC600); lysates were clarified by centrifugation at 12,000 *g* for 15 min at 4°. The protein concentration in the supernatant was estimated using the Bio-Rad protein assay reagent.

MAPK Assays

MAPK activity was determined by either a filter binding assay [43] or an in-gel kinase assay [44].

FILTER BINDING ASSAY. The reaction mixture contained 5 μg of extract protein, and the reaction buffer containing 50 mM β-glycerophosphate, 1.5 mM EGTA, 0.1 mM Na_3VO_4, 1 mM dithiothreitol, 10 mM $MgCl_2$, 20 μM calmidazolium (calmodulin kinase inhibitor), 2 μM PKI (protein kinase A inhibitor), 0.5 mg/mL MBP, 10 μM cold ATP and 2.5 μCi [γ-^{32}P]ATP (3000 Ci/mmol) in a final volume of 25 μL. The reaction was performed at 30° for 15 min and terminated by spotting onto Whatman P81 papers (1-inch squares), washed four times for 15 min each with 400 mL of 150 mM phosphoric acid, pH 7.1; four times for 10 min each with 500 mL of water; and finally one time with acetone for 5 min. The filters were counted in 10 mL of Scintiverse fluid (Fisher Scientific, Pittsburgh, PA) in a Beckman liquid scintillation counter. The specific phosphorylation of MBP was obtained by subtracting the counts from reactions performed without MBP.

IN-GEL KINASE ASSAYS. These were performed *in situ* essentially as described. Briefly, equal amounts of extracts (25 μg) were fractionated on SDS–PAGE gels (10%) containing MBP (0.5 mg/mL), and SDS was removed by incubation in 50 mM Tris, pH 8.0, containing 20% isopropanol for 1 hr at room temperature. Subsequently, proteins on the gel were denatured in 6 M guanidinium HCl and renatured overnight at 4° in 50 mM Tris, pH 8.0, containing 50 mM β-mercaptoethanol and 0.1% Triton X-100 with several changes of buffer. The gel was incubated in kinase buffer containing 40 mM HEPES, pH 8.0, 1.5 mM EGTA, 40 μM ATP, 10 mM $MgCl_2$, 2 mM dithiothreitol and [γ-^{32}P]ATP (5 μCi/mL, 3000 Ci/mmol). The gel was washed with 5% trichloroacetic acid containing 1% sodium pyrophosphate, dried, and exposed to an X-ray film.

Immunoprecipitation of MAPK

The cells were stimulated, washed with cold PBS, and lysed in 10 mM Tris, pH 7.4, containing 1% SDS. The lysates were boiled for 5 min and sonicated for 5 sec to reduce the viscosity. Extracts containing 200 μg of protein were diluted with immunoprecipitation buffer to give a final concentration of 25 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% NP-40, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium-orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine, and 10 μg/mL each of aprotinin and leupeptin. Diluted extracts were immunoprecipitated for 12–16 hr with 5 μg of a monoclonal antibody raised against the C-terminus of rat $p42^{mapk}$ (amino acids 325–345), which also recognizes $p44^{mapk}$. Next, rabbit anti-mouse IgG (10 μg) was added, and 30 min later immunoprecipitates were collected on protein A-agarose beads. Protein A-agarose beads were washed three times with immunoprecipitation buffer, and proteins were eluted by boiling in Laemmli sample buffer for 5 min. The eluted proteins were electrophoresed on denaturing polyacrylamide gels (10%) containing MBP (0.5 mg/mL). MAPK activity was measured by the in-gel kinase assay as described above.

RESULTS

Effect of Ethanol on Serum-Induced MAPK Activation

We used an embryonic liver cell line, BNLCL2, to investigate whether ethanol can modulate agonist-stimulated MAPK ac-

tivity. The advantage of using this cell line is that the cells are non-transformed, and the results obtained can form the basis for determining the mechanisms of hepatotoxic effects of ethanol. The BNLCL2 cells were grown to 80% confluency and starved in 0.1% FBS with 0–400 mM ethanol for 24 hr. Then the cells were washed to remove ethanol and stimulated with serum (10% FBS) for 5 min, and extracts were prepared as described in Materials and Methods. The MAPK activity was determined by an *in vitro* filter binding assay that measured phosphorylation of MBP. Using 5 μg extract protein, the assay was found to be linear for 15 min at 30°. The MAPK activity was expressed as percent over unstimulated control cells (Fig. 1). The basal (control) activity of the extracts ranged from 800 to 2000 dpm/μg protein, whereas in serum-stimulated cells the range was 1600–3500 dpm/μg protein. A consistent pattern of activation was observed in each experiment. Pretreatment of cells with ethanol alone for 24 hr had no significant effect on basal MAPK activity. However, the serum-induced MAPK activity was potentiated by ethanol in a concentration-dependent manner. In control cells, serum stimulated MAPK activity by 160%, whereas it increased to 415% in cells pretreated with 200 mM ethanol for 24 hr. Thus, ethanol potentiated serum-stimulated MAPK activity by 2.5 times. Hence, in subsequent experiments, we used 200 mM ethanol, a concentration at which a large potentiation was observed and more than 80% of the cells were still attached to the dish. At 400 mM ethanol, about 50% of the cells were detached after 24 hr of exposure.

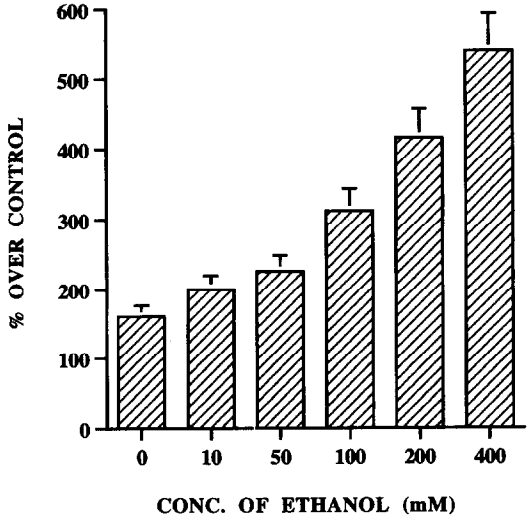

FIG. 1. Effect of different concentrations of ethanol on serum-stimulated MAPK. The BNLCL2 cells were exposed to different concentrations of ethanol (0–400 mM) in DMEM containing 0.1% FBS for 24 hr, washed, and stimulated with serum (10% FBS) for 5 min. The cell extracts were prepared, and 5 μg (protein) of each was assayed for MAPK activity in an *in vitro* filter binding assay, using MBP as substrate (see Materials and Methods.). Data represent the averages of three (± SD) experiments. Basal MAPK activity was in the range of 800–2000 dpm/μg protein in control cells. Values are presented as percent over unstimulated control cells (taken as 100%).

Effects of Ethanol on MAPK Activity Stimulated by PMA, Thrombin, and EGF

To determine whether this effect of ethanol was limited to serum, cells were stimulated with different agonists, serum (FBS, 10%), PMA (160 nM), thrombin (0.5 U/mL), and EGF (60 nM). As shown in Fig. 2, ethanol (200 mM) dramatically potentiated the activation of MAPK by serum (control, 230%; ethanol-treated cells, 480%), PMA (control, 480%; ethanol-treated cells, 810%) and thrombin (control, 480%; ethanol-treated cells, 710%). However, the EGF-stimulated MAPK activity was not affected significantly by ethanol treatment (440 and 560% in control and ethanol-treated cells, respectively). Thus, ethanol potentiated the MAPK activation by agents known to act through different signaling pathways.

Time Course of MAPK Activation in Ethanol-Treated Cells

Whether the potentiation observed in ethanol-treated cells was due to a shift in the time course of MAPK activation also was determined. Control and ethanol-treated (24 hr) cells were stimulated with serum or PMA (160 nM) for different time periods, and MAPK activity of the cytosolic extracts was measured. Serum-stimulated MAPK (Fig. 3A) was highest at 5 min in both control and ethanol-treated cells (200 and 400%, respectively) and declined rapidly to basal level in 30 min. When the cells were stimulated with PMA, the activity of MAPK was highest at 5 min in both control and ethanol-treated cells (2.5- and 4.5-fold, respectively); however, the activity remained at elevated levels for 30 min (Fig. 3B). Thus,

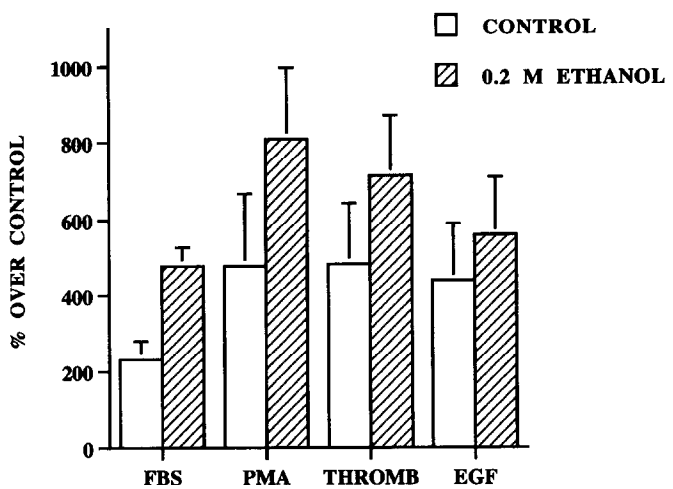

FIG. 2. Effect of ethanol on stimulation of MAPK by serum, PMA, thrombin, and EGF. The figure shows stimulation of MAPK activity (percent over unstimulated control cells) by serum (10% FBS), PMA (160 nM), thrombin (0.5 U/mL), and EGF (60 nM) in the control cells or cells exposed to 200 mM ethanol for 24 hr. The treatment of cells with ethanol, stimulation with agonists, and determination of MAPK activity in the extracts from stimulated cells were as described in Fig. 1. Data are the means ± SD of three separate experiments where each assay was conducted in duplicate. The basal MAPK activity in these experiments was in the range of 1000–2000 dpm/μg protein.

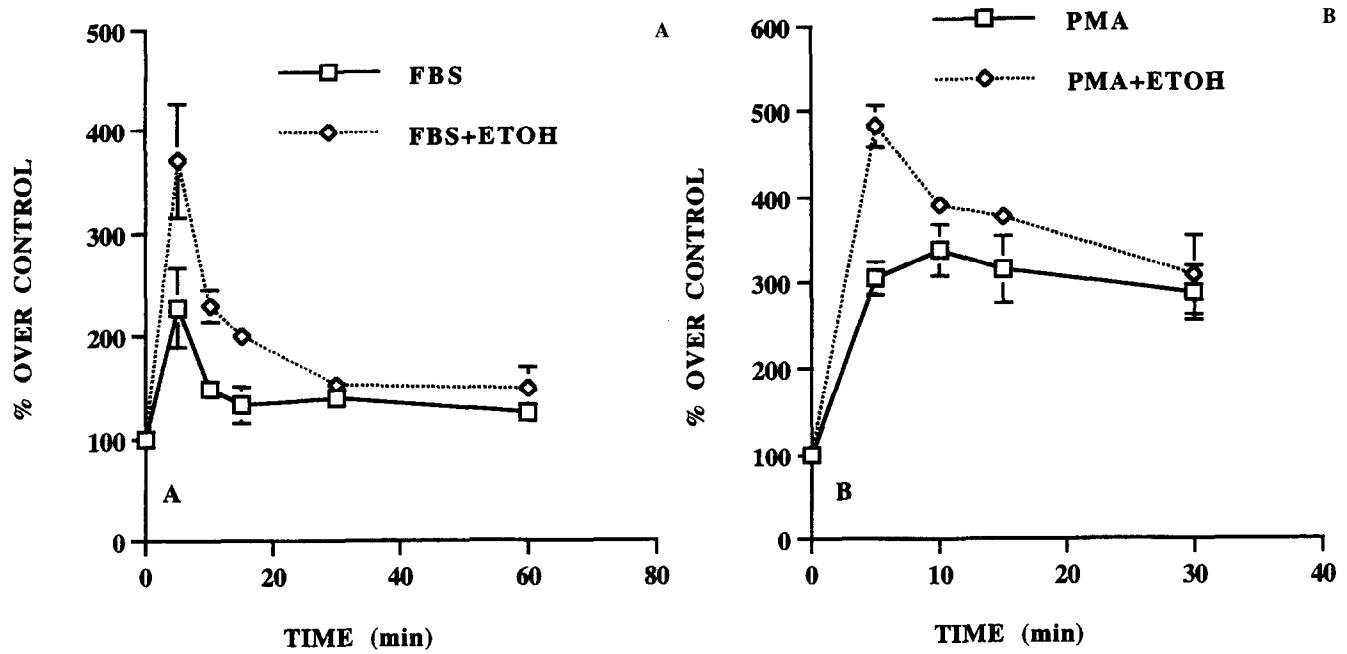

FIG. 3. Time course of MAPK activation by serum (A) and PMA (B). BNLCL2 cells were serum-starved for 24 hr in DMEM containing 0.1% FBS with or without 200 mM ethanol. These cells were stimulated with serum (10% FBS) or PMA (160 nM) for the indicated times, and MAPK activity of cytosolic extracts was determined by an *in vitro* filter binding assay as described in Materials and Methods. Data represent means ± SD (three experiments). The MAPK activity in control cells was in the range of 1000–1800 dpm/μg protein.

the potentiation observed in ethanol-treated cells was not due to alteration in the time course of MAPK activation.

Potentiation of Agonist-Stimulated p42 and p44 MAPK Activity by Ethanol

To verify the results obtained with filter binding assays and to identify which isoforms of MAPK are activated, cytosolic extracts were subjected to an in-gel kinase assay. The cytosolic extracts were fractionated on denaturing polyacrylamide (10%) gels containing MBP (0.5 mg/mL), and the activity of renatured proteins was determined by an in-gel kinase assay [44]. The autoradiogram of a representative experiment is shown in the left panel of Fig. 4. Activities of two kinases in the molecular mass range of 42 and 44 kDa were stimulated in cells treated with serum, PMA, thrombin, and EGF (lane 3, 5, 7, and 9, respectively) as compared with untreated cells (lane 1). Exposure of cells to 200 mM ethanol potentiated the activity of these kinases in cells stimulated with serum, PMA, and thrombin (lanes 4, 6, and 8, respectively) but not with EGF (lane 10). Ethanol alone had no effect on these kinases (lane 2); in fact, it slightly decreased MAPK activity in unstimulated cells. The small decrease in MAPK activity by ethanol alone could not be detected by the filter binding assay. Laser densitometer scanning showed that ethanol increased the serum-, PMA- and thrombin-stimulated MAPK activity by 2-fold. Similar results were obtained in two separate experiments. The autoradiogram of the longer exposure showed that MBP was also phosphorylated by other kinases in the high molecular mass range (not shown). However, activity of these kinases was not affected by stimulation with the agonists. The in-gel kinase assay identified that activities of two kinases, 42 and 44 kDa in size, were stimulated after treatment with serum, PMA, and thrombin, and ethanol potentiated this activity. However, the activation of MAPKs by EGF was not affected by ethanol. The molecular masses of these two kinases corresponded to the MAPK isoforms $p42^{mapk}$ and $p44^{mapk}$. Next, we immunoprecipitated cytoplasmic extracts from BNLCL2 cells stimulated with serum and PMA with a monoclonal antibody raised against the C-terminus of the $p42^{mapk}$, which also recognizes $p44^{mapk}$. The immunoprecipitates were subjected to an in-gel kinase assay. The results (Fig. 4, right panel) showed that only two kinases ($p42^{mapk}$ and $p44^{mapk}$) were specifically immunoprecipitated by the MAPK antibody. Both control and ethanol-treated cells had low basal activity (lanes 1 and 2), whereas serum (lane 3) and PMA (lane 5) dramatically increased the activity of the MAPKs. The activation of the MAPKs by both serum (lane 4) and PMA (lane 6) was potentiated by 200 mM ethanol. Counting the radioactivity in these bands showed a 1.8- and 2-fold potentiation for serum and PMA, respectively, in ethanol-treated cells compared with control cells.

Immunoprecipitation of extracts from PMA-stimulated cells with non-specific monoclonal antibody raised against a bacterial TrpE::lacZ protein (Oncogene Science, Inc., Uniondale, NY) did not show any kinase activity (lane 7). We subjected the whole cell extracts of both control and ethanol-treated cells to western blotting with MAPK monoclonal antibody to detect the levels of MAPK protein. Using this protocol, we could not find any change in the levels of MAPK protein (data not shown). Therefore, the differences in the activities re-

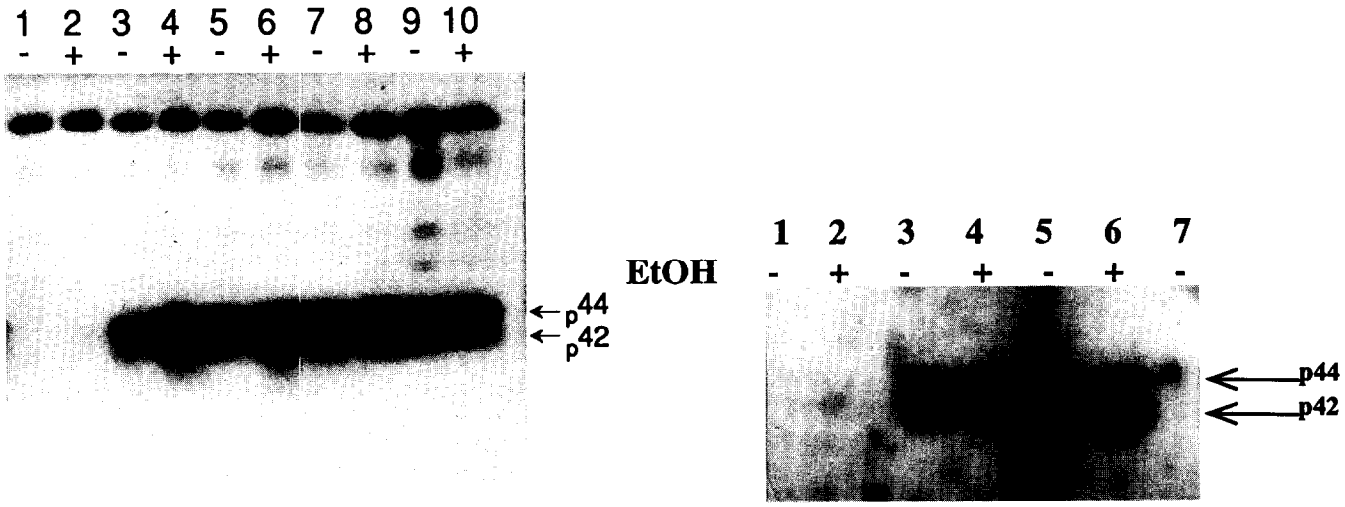

FIG. 4. Effect of ethanol on activation of p42 and p44 MAPK by various agonists. (Left panel) Autoradiogram of in-gel kinase assay performed with total extracts from unstimulated BNLCL2 cells (lanes 1 and 2), or cells stimulated with serum (10% FBS) (lanes 3 and 4), 160 nM PMA (lanes 5 and 6), thrombin, 0.5 U/mL (lanes 7 and 8), and 60 nM EGF (lanes 9 and 10). The (−) and (+) signs above the lanes indicate control cells and cells pretreated with 200 mM ethanol for 24 hr, respectively. The position of $p42^{mapk}$ and $p44^{mapk}$ is indicated by arrows. The extracts made from stimulated cells were separated on 10% denaturing polyacrylamide gels containing MBP (0.5 mg/mL), and in-gel kinase assays of renatured proteins were carried out as described in Materials and Methods. Results are from one of two similar experiments. (Right panel) Immunoprecipitation with MAPK antibody. Autoradiogram of the in-gel kinase assay of immunoprecipitates obtained (using MAPK monoclonal antibody) from the extracts of unstimulated (lanes 1 and 2), serum- (10% FBS) stimulated (lanes 3 and 4) and PMA- (160 nM) stimulated (lanes 5 and 6) liver cells. Lane 7 contains the immunoprecipitate of PMA-stimulated cells with a non-specific monoclonal antibody raised against TrpE::lacZ fusion protein. The signs (−) and (+) above the lanes indicate cells untreated or treated with 200 mM ethanol for 24 hr, respectively. The extracts were immunoprecipitated with a monoclonal antibody raised against the C-terminus of rat $p42^{mapk}$, which also recognizes $p44^{mapk}$, and were measured by the in-gel kinase assay as described in Materials and Methods.

flected the change in the kinase activity and were not due to induction of MAPK kinases.

Involvement of G-Proteins in the Potentiation of MAPK by Ethanol

Several mechanisms including PKC and G-proteins have been proposed to account for the effects of ethanol [8, 22]. To address the involvement of G-proteins, BNLCL2 cells exposed to 200 mM ethanol for 24 hr were treated with PTX (100 ng/mL) for 6 hr prior to stimulation with serum. The extracts from serum-stimulated cells were immunoprecipitated with monoclonal antibody against MAPK, and immunoprecipitates were analyzed by the in-gel kinase assay (Fig. 5, top panel).

The gel slices from the region corresponding to both $p42^{mapk}$ and $p44^{mapk}$ were excised, and radioactivity was counted. The results (Fig. 5, bottom panel) showed that the radioactivity values in serum-stimulated control (no ethanol) and ethanol-treated cells were 270 and 570% over the unstimulated cells. In PTX-treated cells, serum stimulated MAPK activity by 260 and 300% in control and ethanol-treated cells, respectively. Thus, PTX abolished the potentiation by ethanol.

In another series of experiments, control and ethanol-treated cells were pretreated with PTX and stimulated with PMA. Figure 6 shows the autoradiogram of the gel kinase assay performed with total cytosolic extracts. Densitometric scanning of the p44 and p42 MAPK bands showed that PMA stimulated MAPK activity by 3- and 5-fold in control (lane 3) and ethanol-treated cells (lane 4), respectively. In PTX-pretreated cells, PMA stimulated MAPK activity by 3-fold in both control (lane 5) and ethanol-treated cells (lane 6). As shown for serum, PMA-stimulated MAPK activity was potentiated by ethanol, and this effect was abolished by pretreatment with PTX. These results demonstrated that potentiation of activation of MAPK by ethanol is mediated by PTX-sensitive G-protein(s).

DISCUSSION

In this paper, it was demonstrated that chronic treatment of embryonic liver cells with ethanol potentiated MAPK activation by several agents, i.e. serum, PMA, and thrombin, whereas EGF-stimulated MAPK activation was unaffected. Acute or chronic treatment with ethanol had no effect on the basal activity of MAPK. Short-term exposure of liver cells to ethanol (up to 400 mM) had no significant effect on MAPK activation by serum (data not shown). The mechanism by which ethanol exerts its effect on MAPK is not known. Other kinases, such as PKC, cyclic AMP-dependent PKA (protein kinase A), and Ca^{2+}-calmodulin-dependent kinase, are not directly affected by ethanol [45]. In the present study, the addition of ethanol (up to 1 M) to cytosolic extracts prepared from unstimulated cells or from cells stimulated with serum did not cause any change in MAPK activity (data not shown). This indicated that ethanol had no effect on the soluble (cy-

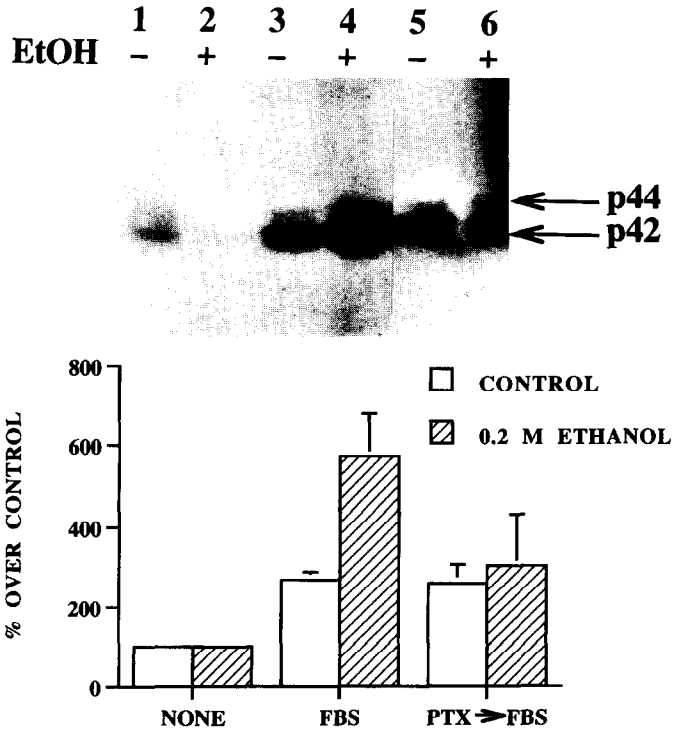

FIG. 5. Effect of PTX on ethanol potentiation of MAPK: Studies in serum-stimulated cells. (Top panel) Autoradiogram of the in-gel kinase assay carried out with immunoprecipitates of extracts from BNLCL2 cells: untreated (lanes 3 and 4) or pretreated with PTX, 100 ng/mL (lanes 5 and 6) and stimulated with serum. Lanes 1 and 2 contain extracts from unstimulated cells. The (−) and (+) signs above the lanes indicate control cells or cells treated with 200 mM ethanol for 24 hr, respectively. The BNLCL2 cells (control or treated with 200 mM ethanol for 24 hr) were pretreated with PTX (100 ng/mL) for 6 hr prior to stimulation with serum. The cells were stimulated with serum (10% FBS) for 5 min, and in-gel kinase assay of immunoprecipitates was performed as described in Fig. 4. The position of p42/p44^{mapk} is indicated by the arrows. (Bottom panel) Radioactivity of the bands corresponding to p42 and p44 MAPK bands was counted and expressed as percent over unstimulated cells. The radioactivity of MAPK bands from unstimulated cells (lanes 1 and 2) was 100 ± 50 dpm in three experiments. PTX → FBS indicates that cells were pretreated with PTX, and were then challenged with serum (10% FBS). Data represent the means ± SD of values from three experiments.

tosolic) MAPK *in vitro*. It also suggested that intact cells are required for the ethanol effect on MAPK and, therefore, the potentiation may likely be due to alteration in signal transduction pathways in cells leading to activation or decrease in the inactivation of MAPKs. Activity of MAPKs may also be regulated by protein tyrosine phosphatases [46]. Several protein phosphatases have been identified with dual specificity towards protein tyrosine and serine/threonine phosphates. These phosphatases can be inhibited by sodium-orthovanadate. When BNLCL2 cells were pretreated with 1 mM sodium-orthovanadate for 1 hr, ethanol potentiation of serum-stimulated MAPK was not affected (data not shown). Further, the activity of protein tyrosine phosphatases was determined by measuring the rate of dephosphorylation of insulin receptor

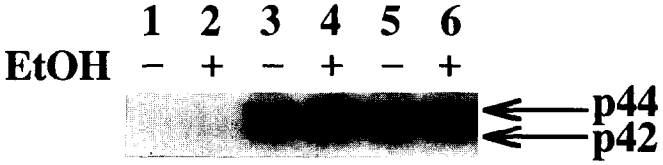

FIG. 6. Effect of PTX on ethanol potentiation of MAPK in PMA-stimulated liver cells. An autoradiogram of the in-gel kinase assay performed with the cytosolic extracts of PMA-stimulated BNLCL2 cells: untreated (lanes 3 and 4) and PTX-treated (lanes 5 and 6). Lanes 1 and 2 contain extracts from unstimulated cells. Pretreatment with PTX (100 ng/mL for 6 hr), stimulation with PMA, preparation of extracts, and in-gel kinase assays were performed as described in the legend to Fig. 4. The position of p44^{mapk} and p42^{mapk} is indicated by the arrows. The signs (+) and (−) indicate with or without ethanol (200 mM) treatment for 24 hr, respectively.

substrate-1 (IRS-1), labeled with ^{32}P on tyrosine residues, in an *in vitro* assay. We could not find any difference in the phosphatase activity between the control and ethanol-treated cells, in either unstimulated or serum-stimulated cells. Thus, inhibition of phosphatases by ethanol was ruled out as a mechanism for MAPK potentiation.

The effect of ethanol on agents acting through diverse pathways indicated that ethanol may exert its action through a common intermediate. Studies in liver and brain cells have shown that both G-proteins and PKC are involved in mediating the effects of ethanol. Our results demonstrate that PTX-sensitive G-protein(s) is needed for ethanol potentiation of MAPK activity in embryonic liver cells. G-proteins coupled to several second messenger systems have been shown to be affected by ethanol. In neuroblastoma cells, acute doses of ethanol activated adenylate cyclase, whereas in cells chronically treated with ethanol, adenylate cyclase and G-protein-coupled PLC activation was inhibited [9, 20]. In hepatocytes, erythrocytes, and platelets, acute treatment with ethanol activates PLC [8]. These effects are considered to be due to alterations in the function of G-proteins. The differential effect of ethanol on different cells may be due to variations in the nature of G-protein subunits, which may be cell specific [20]. The mechanism of action of ethanol on G-proteins is not understood clearly. Ethanol has been proposed to act both at the level of function and expression of G-proteins [9]. The longer time required for the effect of ethanol in BNLCL2 cells favors the speculation that changes in expression of G-proteins may be involved. Recently, it has been demonstrated that alcohol causes an increase in the expression of $G_{i\alpha}$ subunits, whereas it inhibits the expression of $G_{s\alpha}$ subunits in regenerating rat liver [19]. In neuronal cultures, a phosducin-like protein has been shown to be induced by ethanol treatment [47]. Phosducin is a phosphoprotein and is known to modulate G-protein function by binding to $\beta\gamma$ subunits [48]. Phosducin-like protein may also bind to $\beta\gamma$ subunits and modulate G-protein functions. It would be interesting to test whether similar changes take place in BNLCL2 cells. Involvement of PTX-sensitive G-proteins in MAPK activation suggests a potential role for G_i-proteins. Activation of G_i-proteins by agonists like thrombin, LPA or by oncogenic mutation leading to GTPase-defi-

cient protein results in activation of MAPK [35]. Ethanol may act as a weak agonist to stimulate G-proteins, which, in turn, may sensitize the pathways activated by other agonists. It may be noted that ethanol did not affect the EGF-stimulated MAPK. In contrast, it has been reported that ethanol attenuates autophosphorylation of EGF-receptor and phosphorylation of PLC-γ [12, 13]. Thus, the effects of ethanol on tyrosine kinase and MAPKs are distinct.

In summary, we have demonstrated for the first time that ethanol potentiated MAPK activation in fetal liver cells, and PTX-sensitive G-protein pathways appear to be involved in the mechanism. The mechanism of ethanol action involving G-proteins in liver cells is distinct from the one recently described in cultured neuronal cells (PC12). In PC12 cells, ethanol enhances the nerve growth factor-induced MAPK activation by a PKC-dependent mechanism [49]. Potentiation of MAPK activation may have bearing upon normal physiological responses of cells. Some of the proteins phosphorylated by MAPK include phospholipase A_2, c-jun, ribosomal S6 kinase, EGF, and c-myc [42]. Activation of phospholipase A_2 leads to increased arachidonic acid production, which induces inflammatory responses in cells [50]. It also has been shown that c-myc plays an important role in the processes leading to both cell proliferation and apoptosis [51]. Relevance of the potentiation of MAPK by ethanol to the above cellular responses awaits further investigation.

The authors are grateful to Dr. Peter Wilden for his gift of ^{32}P-labeled IRS-1 substrate for the phosphatase assays, to Dr. Bimal Ray for the BNLCL2 cells, and to Ms. Judy Richey for word processing. This work was supported by NIH Grant AA09651.

References

1. Leiber CS, Interactions of ethanol with drugs, hepatoxic agents, carcinogens, and vitamins. *Alcohol Alcohol* **25:** 157–176, 1985.
2. Leiber CS, Biochemical and molecular basis of alcohol induced injury to liver and other tissues. *N Eng J Med* **319:** 1639–1650, 1988.
3. Carter EA and Wands JR, Ethanol induced inhibition of liver cell function. I. Effect of ethanol on hormone stimulated hepatocyte DNA synthesis and the role of ethanol metabolism. *Alcohol Clin Exp Res* **12:** 555–562, 1988.
4. Wands JR, Carter EA, Bucher NLR and Isselbacher KJ, Inhibition of hepatic regeneration in rats by acute and chronic ethanol intoxication. *Gastroenterology* **77:** 528–531, 1979.
5. Dugay L, Couter D, Hetu C and Joly JG, Inhibition of liver regeneration by chronic alcohol administration. *Gut* **23:** 8–13, 1982.
6. Frank WO, Rayes AN, Washington A and Holt PR, Effect of acute ethanol administration upon hepatic regeneration. *J Lab Clin Med* **93:** 402–413, 1982.
7. Poso H, Vaananen H, Salaspuro MP and Poso AR, Effects of ethanol on liver regeneration after partial hepatectomy in rats. *Med Biol* **58:** 329–336, 1980.
8. Hoek JB, Thomas AP, Rooney TA, Higashi K and Rubin E, Ethanol and signal transduction in the liver. *FASEB J* **6:** 2386–2396, 1992.
9. Hoffman PL and Tabakoff B, Ethanol and guanine nucleotide binding proteins: A selective interaction. *FASEB J* **4:** 2612–2622, 1990.
10. Resnicoff M, Sell C, Ambrose D, Baserge R and Rubin R, Ethanol inhibits the autophosphorylation of the insulin-like growth factor 1 (IGF-1) receptor and IGF-2-mediated proliferation of 3T3 cells. *J Biol Chem* **268:** 21777–21782, 1993.
11. Wang S, Jacober L, Wang C and Slomiany BL, Ethanol-induced structural and functional alterations of epidermal growth factor receptor in buccal mucosa. *Int J Biochem* **24:** 85–90, 1992.
12. Thurston AW and Shukla SD, Ethanol modulates basal and epidermal growth factor (EGF) stimulated tyrosine kinase in A431 membranes. *Alcohol & Alcoholism* **27:** 68–72, 1992.
13. Thurston AW and Shukla SD, Ethanol modulates epidermal growth factor stimulated tyrosine kinase and phosphorylation of PLCγ1. *Biochem Biophys Res Commun* **185:** 1062–1068, 1992.
14. Kruger H, Wike PA and Shanley BC, Ethanol and protein kinase C in rat brain. *Neurochem Int* **22:** 575–581, 1993.
15. Depetrillo PB and Liou CS, Ethanol exposure increases protein kinase C activity in human lymphocytes. *Alcoholism* **17:** 351–354, 1993.
16. Roivainen R, Hundle B and Messing RO, Protein kinase C and adaptation to ethanol. *Experientia (Suppl)* **71:** 29–38, 1994.
17. Hoek JB, Thomas AP, Rubin R and Rubin E, Ethanol induced mobilization of calcium by activation of phosphoinositide specific phospholipase C in intact rat hepatocytes. *J Biol Chem* **262:** 682–691, 1987.
18. Higashi K and Hoek JB, Ethanol causes desensitization of receptor-mediated phospholipase C activation in isolated hepatocytes. *J Biol Chem* **266:** 2178–2190, 1991.
19. Diehl AM, Yang SQ, Cole P and Wand GS, Chronic ethanol consumption disturbs G-protein expression and inhibits cyclic AMP-dependent signaling in regenerating rat liver. *Hepatology* **16:** 1212–1219, 1993.
20. Simonsson P, Rodriguez FD, Loman N and Alling C, G proteins coupled to phospholipase C: Molecular targets of long-term ethanol exposure. *J Neurochem* **56:** 2018–2026, 1991.
21. Mochly-Rosen D, Chang F-H, Cheever L, Kim M, Diamond I and Gordon AS, Chronic ethanol causes heterologous desensitization by reducing α_s mRNA. *Nature* **333:** 848–850, 1988.
22. Messing RO, Petersen PJ and Henrich CJ, Chronic ethanol exposure increases levels of protein kinase C δ and ε and protein kinase C-mediated phosphorylation in cultured neural cells. *J Biol Chem* **266:** 23428–23432, 1991.
23. Rubin R and Hoek JB, Alcohol induced stimulation of phospholipase C in human platelets requires G-protein activation. *Biochem J* **254:** 147–153, 1988.
24. Michalopoulous GK, Liver regeneration: Molecular mechanisms of growth control. *FASEB J* **4:** 176–187, 1991.
25. Erikson RL, Structure, expression and regulation of protein kinases involved in the phosphorylation of ribosomal protein S6. *J Biol Chem* **266:** 6007–6010, 1991.
26. Cobb MH, Boulton TG and Robbins DJ, Extracellular regulated kinases: ERKs in progress. *Cell Regul* **2:** 965–978, 1991.
27. Pelech SL and Sanghera JS, Mitogen activated protein kinases: Versatile transducers for cell signalling. *Trends Biochem Sci* **17:** 233–238, 1992.
28. Blenis J, Signal transduction via the MAP kinases: Proceed at your own RSK. *Proc Natl Acad Sci USA* **90:** 5889–5892, 1993.
29. Boulton TG, Nye SH, Robbins DJ, Ip NY, Radziejewska E, Morgenbesser SD, DePinho RA, Panayatotos N, Cobb MH and Yancopoulos, GD, ERKs: A family of protein/serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. *Cell* **65:** 663–675, 1991.
30. Egan SE, Giddings BW, Brooks MW, Buday L, Sizeland AM and Weinberg RA, Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. *Nature* **363:** 45–51, 1993.
31. McCormick F, How receptors turn Ras on. *Nature* **363:** 15–16, 1993.
32. Dent P, Haser W, Haystead TAJ, Vincent LA, Roberts TM and Sturgill TW, Activation of mitogen-activated protein kinase ki-

nase by v-Raf in NIH 3T3 cells and *in vitro*. *Science* **257:** 1404–1407, 1992.
33. Kyriakis JM, App H, Zhang X-f, Banerjee P, Brautigan DL, Rapp UR and Avruch J, Raf-1 activates MAP kinase-kinase. *Nature* **358:** 417–421, 1992.
34. Crews CM, Alessandrini A and Erikson RL, The primary structure of MEK, a protein kinase that phosphorylates the *ERK* gene product. *Science* **258:** 478–480, 1992.
35. Blumer KJ and Johnson GL, Diversity in function and regulation of MAP kinase pathways. *Trends Biochem Sci* **19:** 236–240, 1994.
36. Crews CM and Erikson RL, Extracellular signals and reversible protein phosphorylation: What to Mek of it al. *Cell* **74:** 215–217, 1993.
37. Winitz S, Russell M, Qian N-X, Gardner A, Dwyer L and Johnson GL, Involvement of Ras and Raf in the G_i-coupled acetylcholine muscarinic m2 receptor activation of mitogen-activated protein (MAP) kinase kinase and MAP kinase. *J Biol Chem* **268:** 19196–19199, 1993.
38. Alblas J, van Corven EJ, Hordijk PL, Milligan G and Moolenaar WH, G_i-mediated activation of the $p21^{ras}$-mitogen-activated protein kinase pathway by α_2-adrenergic receptors expressed in fibroblasts. *J Biol Chem* **268:** 22235–22238, 1993.
39. Lange-Carter CA, Pleiman CM, Gardner AM, Blumer KJ and Johnson GL, A divergence in the MAP kinase regulatory network defined by MEK kinase and raf. *Science* **260:** 315–319, 1993.
40. Hordijk PL, Verlaan I, van Corven EJ and Moolenaar WH, Protein tyrosine phosphorylation induced by lysophosphatidic acid in rat-1 fibroblasts. Evidence that phosphorylation of MAP kinase is mediated by the G_i-$p21^{ras}$ pathway. *J Biol Chem* **269:** 645–651, 1994.
41. Crespo P, Xu N, Simonds WF and Gutkind JS, Ras-dependent activation of MAP kinase pathway mediated by G-protein $\beta\gamma$ subunits. *Nature* **369:** 418–420, 1994.
42. Davis RJ, The mitogen activated protein kinase signal transduction pathway. *J Biol Chem* **268:** 14553–14556, 1993.
43. Ahn NG, Weiel JE, Chan CP and Krebs EG, Identification of multiple epidermal growth factor-stimulated protein serine/threonine kinases from Swiss 3T3 cells. *J Biol Chem* **265:** 11487–14494, 1990.
44. Gotoh Y, Nishida E, Yamashita T, Hoshi M, Kawasaki M and Sakai H, Microtubule-associated-protein (MAP) kinase activated by nerve growth factor and epidermal growth factor in PC12 cells. *Eur J Biochem* **193:** 661–669, 1990.
45. Machu TK, Olsen RW and Browning MD, Ethanol has no effect on cAMP-dependent protein kinase-, protein kinase C-, or Ca^{2+}-calmodulin-dependent protein kinase II-stimulated phosphorylation of highly purified substrates *in vitro*. *Alcoholism* **15:** 1040–1044, 1991.
46. Nebreda AR, Inactivation of MAP kinases. *Trends Biochem Sci* **19:** 1–2, 1994.
47. Miles MF, Barhite S, Sganga M and Elliott M, Phosducin-like protein: An ethanol-responsive potential modulator of guanine nucleotide binding protein function. *Proc Natl Acad Sci USA* **90:** 10831–10835, 1993.
48. Reig JA and Klein DC, Pineal transduction: Adrenergic → cyclic AMP-dependent phosphorylation of cytoplasmic 33 kDa protein (MEKA) which binds $\beta\gamma$ complex of transducin. *J Biol Chem* **265:** 5816–5824, 1990.
49. Roivainen R, Hundle B and Messing OR, Ethanol enhances growth factor activation of mitogen-activated protein kinases by a protein kinase C-dependent mechanism. *Proc Natl Acad Sci USA* **92:** 1891–1895, 1995.
50. Needleman P, Turk J, Jaschik BA, Morrison AR and Lefkowitz JB, Arachidonic acid and metabolism. *Annu Rev Biochem* **55:** 69–102, 1986.
51. Evan GI and Littlewood TD, The role of c-myc in cell growth. *Current Opin Genet Dev* **3:** 44–49, 1993.