British Journal of Pharmacology (1999) 128, 1761–1771
http://www.stockton-press.co.uk/bjp

Early intracellular signalling pathway of ethanol in vascular smooth muscle cells

*,[1]Agapios Sachinidis, [1]Ioanna Gouni-Berthold, [1]Claudia Seul, [1]Stefan Seewald, [1]Yon Ko, [1]Udo Schmitz & [1]Hans Vetter

[1]Medizinische Universitäts-Poliklinik, Wilhelmstr. 35-37, 53111 Bonn, Germany

1 ERKs belong to MAP kinase family and are activated by several growth and stress factors. Although ethanol has been shown to modulate ERK1 and ERK2 ($p44^{mapk}$ and $p42^{mapk}$) activity, it can also act as an antiproliferative agent in various mammalian cells. Since the nature of the antiproliferative effect of ethanol in VSMCs has not been defined, we examined its effects on growth and on early intracellular events normally induced by growth factors in VSMCs.

2 Measurement of cytosolic Ca^{2+} and pH in cell monolayers was performed using fura-2/AM and BCECF/AM, respectively. The effect of ethanol on VSMCs growth was assessed by [^{3}H]-thymidine incorporation, by cell counting and by determination of the caspase 3 activity. Stimulation of ERK1 and ERK2 was examined by the chemiluminescence Western blotting method. The expression of c-fos was quantitated by Northern blotting. Determination of inositolphosphates was performed after labelling of VSMCs with myo-[2-^{3}H]-inositol and separation of inositolphosphates by HPLC.

3 Ethanol (0.3–1.0% v v^{-1}, 17–170 mM) induced a dose-dependent maximal stimulation of $p44^{mapk}/p42^{mapk}$ at 30 min and expression of c-fos mRNA with a maximum at 120 min. Intracellular events upstream to MAP kinase, like an increase in $[Ca^{2+}]_i$, activation of the Na^+/H^+ exchanger and formation of phosphoinositol metabolites were also markedly activated by ethanol. Treatment of VSMCs with ethanol for 3–5 min induced an increase in DNA synthesis whereas treatment of the cells for more than 30 min was toxic. Caspase 3 activity was not modulated by ethanol treatment of VSMCs.

4 We may postulate that the activation of these mitogenic signals including the elevation of DNA synthesis reflects a cell effort to protect itself against the toxic effects of ethanol.

Keywords: Ethanol; MAP kinase; c-fos; inositolphosphates; smooth muscle cells

Abbreviations: Ang II, Angiotensin II; BCECF, 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein; DMEM, Dulbecco's Modified Eagle's medium; ERKs, extracellular response kinases; FCS, foetal calf serum; fura-2/AM, fura-2-pentaacetoxymethyl ester; GAP, GTPase activating protein; G_i protein, GTP binding protein; GTP, guanosine triphosphate; $InsP_1$, inositol 1-phosphate; $InsP_2$, inositol 1,4-bisphosphate; $InsP_3$, inositol-1,4,5-phosphate; MAP, mitogenic-activated protein; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; PI-3, phosphatidylinositol-3; PLC, phospholipase C; PTX, pertussis toxin; Raf-1, a 74 kDa protein kinase encoded by the proto-oncogene raf-1; VSMCs, vascular smooth muscle cells

Introduction

It is widely believed that growth factors such as the platelet-derived growth factor (PDGF) or angiotensin II (Ang II) play a pivotal role in the development of hypertension and atherosclerosis by promoting VSMC growth (Daemen *et al*., 1991; Ross, 1993). The early intracellular signalling pathway of PDGF or Ang II in VSMCs leading to VSMC growth has been extensively studied. Binding of Ang II or PDGF-BB to its respective receptors stimulates phosphoinositide catabolism, elevates intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), stimulates the Na^+/H^+ exchange, and mitogen-activated protein (MAP) kinases as well as induces the expression of immediate early growth response genes in VSMCs (Abedi & Zachary, 1995; Duff *et al*., 1995; Sachinidis *et al*., 1993; Timmermans *et al*., 1993). Finally, both agonists promote growth and contraction of VSMCs (Abedi & Zachary, 1995; Timmermans *et al*., 1993). Activation of MAP kinase family members such as the extracellular response kinases (ERKs) and the c-Jun amino-terminal kinases (JNKs) occurs not only by the effect of diverse stimuli including hormones and growth factors but also by stress caused through hypoxia, oxidative stress, osmotic imbalance, heat shock, inhibition of protein synthesis and irradiation (Fanger *et al*., 1997; Robinson & Cobb, 1997) in various cell types. Also, stress factors such as hypoxia or ethanol induce expression of the heat shock proteins (HSP) HSP70 and HSP28 in mammalian cells (Hahn *et al*., 1991; Milarsky & Morimoto, 1986). In this context, it has been postulated that determination of growth, differentiation and programmed cell death (apoptosis) may be associated with the activity of MAPK family members and that the balance of their activity is critical in determining cell fate (Fanger *et al*., 1997; Robinson & Cobb, 1997). The best-characterized ERKs are ERK1 and ERK2 which are proteins with a molecular weight of 44 and 42 kDa ($p44^{mapk}/p42^{mapk}$), respectively. Recently, it has been demonstrated that like growth factors, ethanol is able to induce contraction of smooth muscles dependent on an elevation in $[Ca^{2+}]_i$ (Zheng *et al*., 1997). Remarkably, 100 mM ethanol causes a phospholipase C (PLC)-dependent elevation of $InsP_3$ and Ca^{2+} mobilization in rat hepatocytes (Hoek *et al*., 1990). Furthermore, treatment of rat hepatocytes with 100 mM ethanol *in vitro* for 16 h prolonged the activation of $p44^{mapk}/p42^{mapk}$ induced by various growth factors (Chen *et al*., 1998). However, proliferation of the cells was inhibited (Chen *et al*., 1998). Also, acute

*Author for correspondence; E-mail: sachinidis@uni-bonn.de

treatment of rat hepatocytes with 50 mM ethanol elevated basal activity of the $p42^{mapk}$ and prolonged the nerve growth factor (NGF)-induced activity of the $p42^{mapk}$ (Tombes *et al*., 1998). On the one hand ethanol has been shown to modulate intracellular signalling events normally induced by growth factors. On the other hand, ethanol acts as an antiproliferative agent in several cell types including hepatocytes (Chen *et al*., 1998; Tombes *et al*., 1998) glia cells (Luo & Miller, 1997) and VSMCs (Hendrickson *et al*., 1998). Excessive proliferation of arterial vascular smooth muscle cells (VSMCs) plays an important role in the process of atherogenesis and hypertension (Cho *et al*., 1997; Dzau & Gibbons, 1993; Ross, 1993). Therefore, inhibition of VSMC proliferation may be crucial for preventing development of cardiovascular diseases. In order to prevent restenosis, it has been demonstrated that local delivery of 15% ethanol solution to pig coronary arteries significantly decreased smooth muscle proliferative activity and neointima formation induced by balloon dilatation injury (Liu *et al*., 1997). To explain the inhibitory effect of ethanol on VSMCs proliferation, the authors speculated that ethanol may lead to an alteration of membrane receptors which reduces the cellular responses to growth stimulants (Liu *et al*., 1997). However, the nature of the antiproliferative effect of ethanol and its early intracellular signalling transduction pathway in VSMCs has not been defined. Therefore, we examined the effects of ethanol on VSMC growth by different proliferation assays and by testing for apoptosis. Furthermore, we examined the effects of ethanol on the early intracellular signalling transduction pathway in VSMCs.

Methods

Isolation and culture of vascular smooth muscle cells

Rat aortic VSMCs were isolated from thoracic aortas of Wistar-Kyoto rats (6–8 weeks old, Charles River Wiga GmbH) by enzymatic dispersion using a slight modification of the method of Chamley *et al*. (1979) as described previously (Sachinidis *et al*., 1995). Cells were cultured in DMEM supplemented with 10% FCS (v v^{-1}), nonessential amino acids, penicillin 100 IU ml^{-1} and 6.8 μM streptomycin at 37°C in the Steri-cult incubator (Forma Scientific, Göttingen, Germany) in a humidified atmosphere of 95% air and 5% CO_2. Cells (3×10^6) were grown in 75 cm^2 flasks to confluence over 4–5 days. The purity of VSMC cultures was confirmed by immunocytochemical localization of smooth muscle specific α-smooth muscle actin. Experiments were performed using cells between passages 5 to 20.

Measurement of $[Ca^{2+}]_i$

VSMCs were cultured on round glass microscope slides (diameter 12 mm) under normal tissue culture conditions until confluence. Cells were incubated with 2 μM fura-2 pentaacetoxymethyl ester at 37°C for 20 min in N-(2-hydroxyethyl)piperazine-N′-2-ethanesulphonic acid (HEPES) buffer (in mM: 20 HEPES, 16 glucose, 130 NaCl, 1 $MgSO_4$, 7 H_2O, 0.5 $CaCl_2$, Tris-base, pH 7.4) supplemented with 1% bovine serum albumin (BSA) (w v^{-1}). Just prior to the measurements, the cell monolayer was rinsed with HEPES buffer without BSA, containing 1 mM $CaCl_2$, and the glass slide was positioned diagonally in the cuvette. Measurements were performed in HEPES buffer containing 1 mM $CaCl_2$. Measurements in the absence of extracellular Ca^{2+} were performed in HEPES buffer without $CaCl_2$ containing 1 mM EGTA. The Ca^{2+}-fura-2 fluorescence was measured at 37°C in a Perkin-Elmer LS50 fluorescence spectrofluorometer at excitation wavelengths of 340 and 380 nm and at an emission wavelength of 505 nm (Grynkiewicz *et al*., 1985). After calibration of fluorescence signals, $[Ca^{2+}]_i$ was calculated using the following equation $[Ca^{2+}]_i = K_d \times (R - R_{min})/(R_{max} - R) \times (S_{f2}/S_{b2})$. K_d for the fura-2/Ca^{2+} complex at 37°C is assumed to be 224 nM. S_{f2} is the 380 nm-excited fluorescence in the absence of Ca^{2+} (EGTA added) and S_{b2} is the 380 nm-excited fluorescence in the presence of a saturating Ca^{2+} concentration (1 mM Ca^{2+}).

Measurement of pH_i

For the measurement of pH_i, confluent cells were detached with 0.04% trypsin (w v^{-1}), 0.02% EDTA (w v^{-1}) in Dulbecco's phosphate-buffered saline (PBS) after 5–10 min at 37°C. Then cells were cultured on round glass microscope slides (diameter 12 mm) under normal tissue culture conditions. When they became confluent they were incubated with the fluorescence pH indicator 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF) at a concentration of 4 μM for 20 min at 37°C in HEPES buffer supplemented with 1% BSA (w v^{-1}). Just prior to the measurements, the cell monolayer was rinsed with HEPES buffer and the glass slide was positioned diagonally in the cuvette. Measurements were performed in HEPES buffer without BSA in the LS50 luminescence spectrofluorometer equipped with the fast filter application (Perkin Elmer, Überlingen, Germany). For the fluorescence measurements the following wavelengths were set: excitation wavelengths: 492 and 438 nm; emission wavelength 525 nm. Calibration of BCECF fluorescence was performed in HEPES buffer in which NaCl was replaced by KCl by permeabilizing the cells with the K^+/H^+ ionophore nigericin (1.3 μM) in the presence of 2-(N-morpholino)ethane sulphonic acid (MES) as previously described (Rink *et al*., 1982). The fluorescence of BCECF was approximately linear between pH_i 7.4 and 6.4.

Determination of inositolphosphates

The determination of inositol 1-phosphate ($InsP_1$), inositol 1,4-bisphosphate ($InsP_2$), and inositol 1,4,5-trisphosphate ($InsP_3$) was performed as previously described (Berridge *et al*., 1983). Cells were seeded in petri dishes (60 mm diameter) and grown in the presence of 4 μCi ml^{-1} myo-[2-^{3}H]-inositol for 3 days. During this time the cells reached confluence. They were washed three times with HEPES buffer, followed by a 2 h incubation in DMEM without FCS. After two more washes with buffer, the cells were incubated for 20 min in DMEM containing 20 mM LiCl to inhibit inositol-1 monophosphatase activity. Then they were stimulated with ethanol for different time intervals. The reaction was terminated by the addition of ice-cold 8.0% (w v^{-1}) trichloroacetic acid (TCA). Samples were kept on ice for 30 min before removing the TCA by extracting the samples with diethyl ether. Radiolabelled inositolphosphates were analysed and quantified by standardized anion-exchange high-pressure liquid chromatography (HPLC) (Merck, Hitachi, model 655A-12, Darmstadt, Germany) (Berridge *et al*., 1983). HPLC was performed in an anion exchanger Partisil SAX column using the following elution conditions: Flow rate, 1.25 ml min^{-1} $fraction^{-1}$, 0–6 min, 100% water; 7–30 min a linear gradient from water to 100% 1.0 M ammonium formate/orthophosphoric acid pH 3.7; 31–35 min 100% 1.0 M ammonium formate/orthophosphoric acid pH 3.7; 36–37 min a linear gradient from 1.0 M ammonium formate/orthophosphoric acid pH 3.7 to 100%

water; 38–48 min 100% water. The 1.25 ml fractions were transferred to scintillation vials and radioactivity was determined in a liquid scintillation counter (Beckman LS 3801, Düsseldorf, Germany).

Gel electrophoresis and immunostaining

VSMCs were seeded in 3 cm petri dishes (4×10^5 cells dish^{-1}) and cultivated in culture medium until confluent. The medium was then replaced by serum-free medium consisting of a mixture of DMEM and Ham's F-10 medium (1 : 1, v v^{-1}). Following another 24 h cultivation in serum-free medium, cells were treated with ethanol for different time periods. After removal of the medium cells were lysed with the SDS sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS (w v^{-1}), 10% glycerol (v v^{-1}) and 50 mM dithiothreitol. Aliquots were used for protein determinations using the Bio-Rad protein assay as described previously (Bradford, 1976). Then 0.1% bromphenol blue (w v^{-1}) was added to the aliquots. Thirty μg of protein were analysed by SDS–PAGE in a 10% acrylamide gel with a thickness of 0.75 mm using the Mini Gel Protein system (Bio-Rad). MAP kinase protein analysis was performed by the chemiluminescence Western blotting method as described in the instructions of the PhosphoPlus^{TM} mapk Antibody Kit (New England BioLabs). After the transfer of proteins to a polyvinylidenedifluoride membrane blocking and antibody incubations were performed according to the instruction manual of the PhosphoPlus^{TM} MAPK Antibody Kit (New England BioLabs) using a phospho-specific mapk rabbit polyclonal IgG primary antibody and the alkaline phosphatase-conjugated anti-rabbit secondary antibody. The primary antibody recognized p42^{mapk} and p44^{mapk} only when catalytically activated by phosphorylation at Tyr204 (Marshall, 1995). Chemiluminescence detection of the active p42^{mapk}/p44^{mapk} was performed as described in the instruction manual of the Kit using the Phototope^{TM} Western detection system.

Analysis of c-fos mRNA

The expression of c-fos mRNA was studied in confluent cells in 75 cm^2 flasks starved of serum for 24 h. Then the cells were treated with 1.0% (v v^{-1}, 170 mM) ethanol for different time periods. Medium was aspirated and cells were lysed with 1 ml TRI reagent (Sigma). Total RNA was extracted according to the manufacturer's protocol as described previously (Chomczynski & Sacchi, 1987). Ten μg of total RNA were separated by electrophoresis in a 6% formaldehyde/1.2% agarose gel, blotted on Hybond N$^+$ membranes (Amersham, Little Chalfont, England), washed at room temperature in 5 × SSC (1 × = 0.15 M NaCl, 0.015 M sodium citrate) for 5 min, and fixed with UV irradiation. After fixing, the blots were washed at 60°C in 0.1 × SSC, 0.1% SDS (sodium dodecylsulphate) for 5 min. Prehybridization and hybridization were performed overnight at 60°C in 5 × SSC, 0.2% SDS, 50 mM sodium phosphate, 10 × Denhardt's solution (Sigma Chemical, Deisenhofen, Germany) and 200 μg ml^{-1} salmon sperm (ss)DNA. The DNA probes were labelled with α-^{32}P-deoxycytidine triphosphate (^{32}P-dCTP) by random oligonucleotide priming to a specific activity of $2-4 \times 10^9$ d.p.m. μg^{-1} DNA (Amersham Buchler, Braunschweig, Germany). The stringency of the final wash was 0.2 × SSC containing 0.1% SDS) at 65°C for 2 × 45 min. The Blots were exposed to Kodak films (Kodak X-OMAT, 8 × 10 inch, Rochester, U.S.A.) for 3–7 days at −70°C and were standardized using a 0.77 kb cDNA probe for β-actin (Dianova/Oncor, Hamburg, Germany). The size in kilobases (kb) of the detected mRNA was estimated from the 18S (1.8 kb) and 28S (4.6 kb) ribosomal RNA bands.

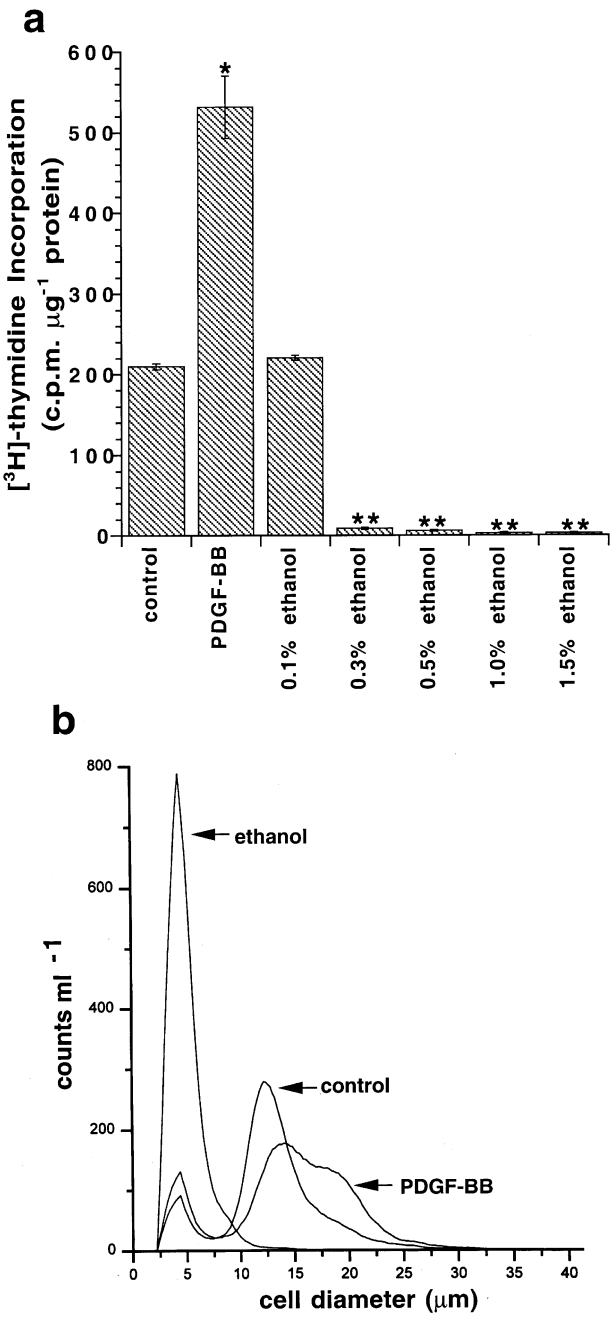

Figure 1 Effect of ethanol on [^{3}H]-thymidine incorporation into VSMC DNA and cell counts. Confluent VSMCs were precultured in 24-well plates for 24 h in serum-free medium. Then cells were treated with different concentrations of ethanol or 50 ng ml^{-1} PDGF-BB. Following another 20 h of incubation, cells were exposed to 3 μCi ml^{-1} [^{3}H]-thymidine. Four hours later the reaction was terminated and [^{3}H]-thymidine incorporation into cell DNA and cell protein (a) were quantified. Results are from a representative experiment from three separate independent experiments, performed in triplicate wells and are expressed as mean ± s.e.mean, $n = 3$, $^*P < 0.05$ for PDGF-BB effect versus control. $^{**}P < 0.05$ for ethanol effect versus control. (b) Confluent VSMCs were precultured in 24-well plates for 24 h in serum-free medium. Then cells were treated with 1.0% ethanol or 50 ng ml^{-1} PDGF-BB. After 24 h cells were trypsinized and cell counts were determined with the cell counter system CASY-1 (Schärfe System) (one representative experiment from three separate independent experiments).

Determination of DNA synthesis

The effect of ethanol on [^{3}H]-thymidine incorporation into cell DNA was assessed as previously described (Sachinidis *et al*., 1995). VSMCs were seeded in 24-well culture plates and grown to confluence. Then the medium was replaced by serum-free medium consisting of a mixture of DMEM and Ham's F-10 medium (1 : 1, v v^{-1}). After 24 h cultivation in serum-free medium, cells were incubated with 1.0% ethanol or 50 ng ml^{-1} PDGF-BB for 20 h before 3 μCi ml^{-1} [^{3}H]-thymidine was added. Four hours later the experiments were terminated by aspirating the medium and subjecting the cultures to sequential washes with PBS containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10% trichloroacetic acid (w v^{-1}) and ethanol/ether (2 : 1, v v^{-1}). Acid insoluble [^{3}H]-thymidine was extracted using 250 μl well^{-1} of 0.5 M NaOH and 100 μl of this solution was mixed with 5 ml scintillator liquid (Packard, Ultimagold) and quantified using a liquid scintillation counter, model Beckman LS 3801, Düsseldorf, Germany. Fifty μl of the residual solution was used for the determination of protein using the Bio-Rad protein assay as described previously (Bradford, 1976). In another set of experiments, after 24 h cultivation in serum-free medium, cells were incubated with 1.0% ethanol for 3, 5, 10, 15, 30, 60 min (after 3, 5, 10, 15, 30 and 60 min incubation the medium was replaced with fresh serum-free medium for a total of 20 h) and 20 h. Then, 3 μCi ml^{-1} [^{3}H]-thymidine was added to the serum-free medium. Four hours later the experiments were terminated as described above.

Determination of cell counts and cell diameter

For cell counting, VSMCs were seeded in 24-well culture plates (5×10^4 cells well^{-1}, well diameter 12 mm) and cultured in DMEM, supplemented with 10% FCS (v v^{-1}), non essential amino acids, penicillin 100 IU ml^{-1} and streptomycin 100 μg ml^{-1} at 37°C for 24 h. Under these conditions, a cell confluence of approximately 70% was reached. The medium was then replaced by serum-free medium consisting of DMEM and Ham's F-10 (1 : 1, v v^{-1}). After 24 h VSMCs were either treated with 1.0% ethanol or stimulated with 50 ng ml^{-1} PDGF-BB. After another 24 h the cells were trypsinized and cell counting as well as determination of cell diameter was performed using the CASY-1 system based on the coulter counter principle (Schärfe, Reutlingen, Germany).

In another set of experiments, VSMCs (approximately 70% confluence) were preincubated in serum-free medium for 24 h. Cells were then treated with 1% ethanol for 3, 5, 10, 15, 30, 60 min and 24 h (after 3, 4, 10, 15, 30 and 60 min, medium was replaced with fresh serum-free medium without ethanol). Cell counting was performed at 24 h using CASY-1.

Apoptosis assay

Apoptosis of VSMCs was determined by the FluoAce^{TM} apopain assay kit from Bio-Rad based on continuous fluorimetric assay of caspase 3 activity as described in the manual of the Kit. Caspase 3 activity was monitored using the fluorogenic peptide substrate carbobenzoxy-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (AFC). Cas-

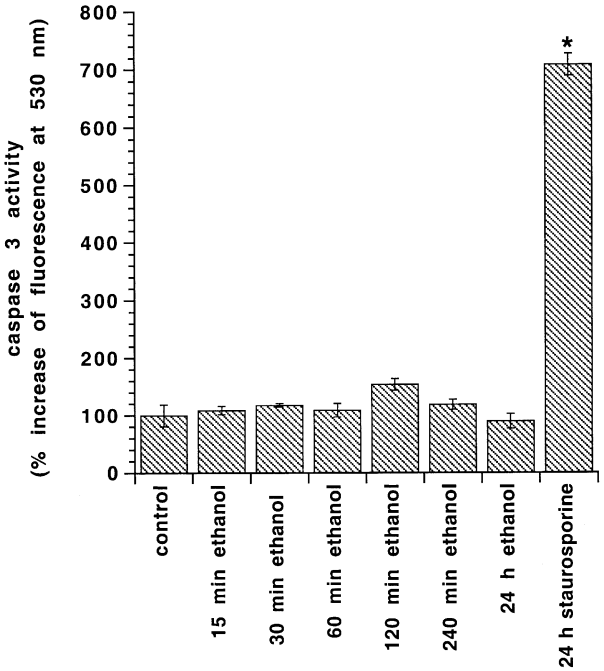

Figure 2 Effect of ethanol on caspase 3 activity in VSMCs. Confluent VSMCs in 10-cm petri dishes were preincubated in 5 ml serum-free medium for 24 h. VSMCs were treated for 15, 30, 60, 120, 180 min and 24 h with 1.0% ethanol or with 100 nM staurosporine for 24 h. Cells were then lyzed and apopain activity was measured in 1 ml reaction buffer containing 1 mM PIPES, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT and 50 μM carbobenzoxy-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin. Fluorescence was measured after 180 min. Caspase 3 activity was expressed as per cent increase of the fluorescence at 180 min (the fluorescence of untreated cells was considered as 100%). Results are from a representative experiment performed in triplicate 10 cm petri dishes and are expressed as mean $\pm$ s.e.mean, $n = 3$, $^*P < 0.05$ for staurosporine effect versus control.

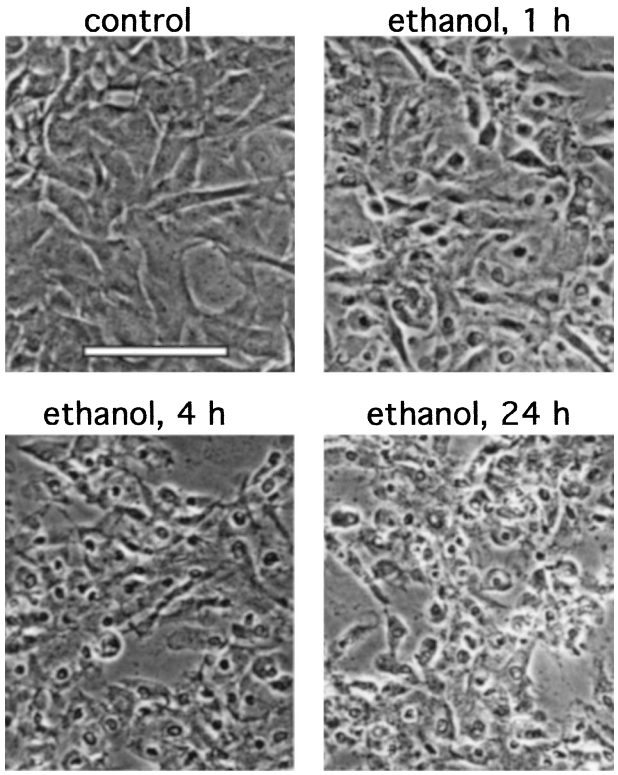

Figure 3 Morphological changes of VSMCs after treatment with 1.0% ethanol. Confluent VSMCs were precultured in 24-well plates for 24 h in serum-free medium. Then cells were treated with 1.0% ethanol for different time periods. Cells were photographed by phase-contrast light microscope. After treatment of the cells with ethanol cells appear more granular and 'blebby'. Calibration bar represents 125 μm.

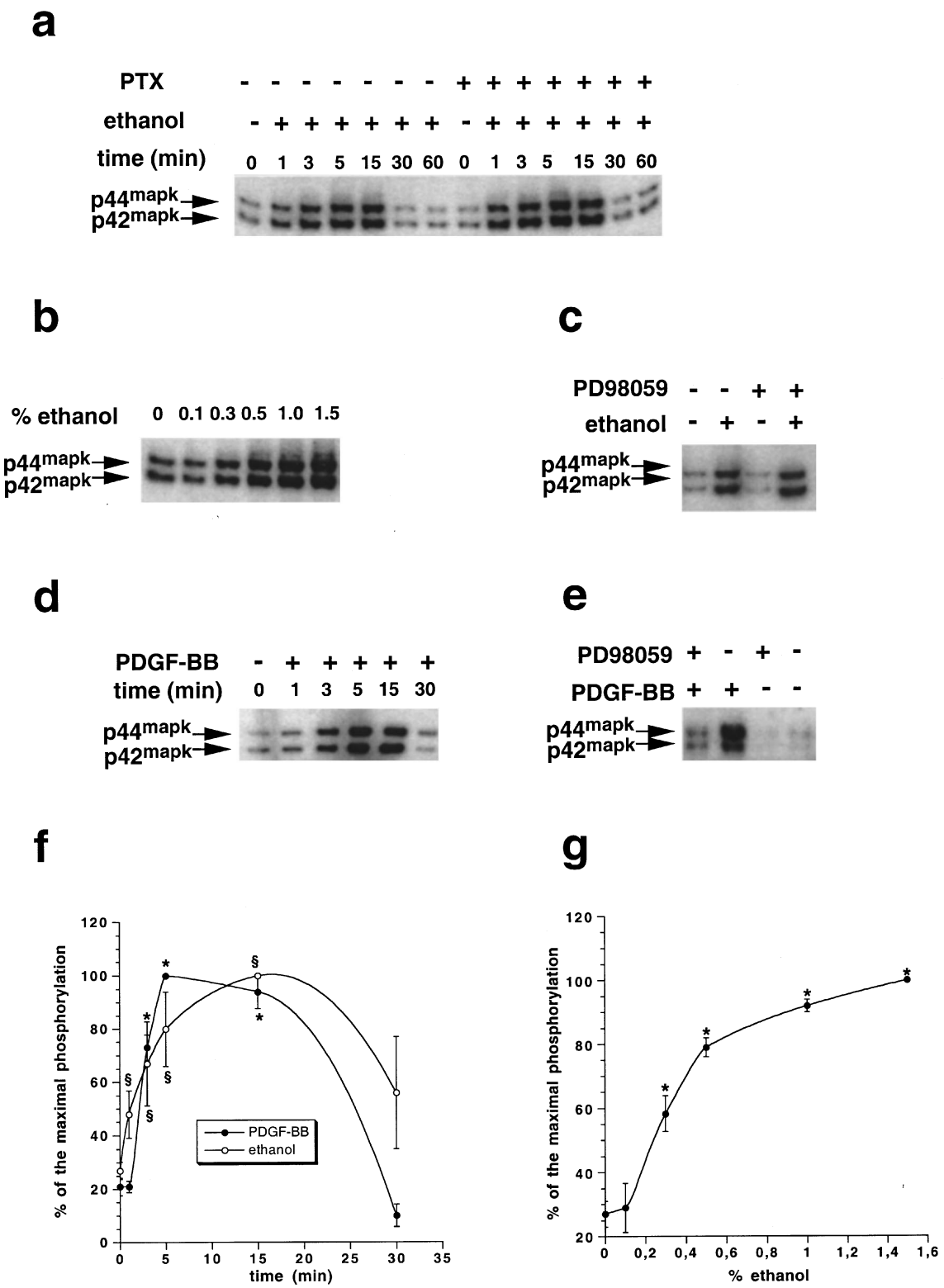

Figure 4 Effect of ethanol on the phosphorylation of the $p44^{mapk}/p42^{mapk}$ at Tyr 204 in VSMCs. (a) VSMCs were seeded in culture dishes (diameter: 3 cm) and cultivated in culture medium until confluence. Then the medium was replaced by serum-free medium. Following 24 h cultivation in the presence and absence of 100 ng ml^{-1} PTX cells were treated with 1.0% ethanol for different time periods or PDGF-BB (50 ng ml^{-1}) for 5 min. Cells were lysed and 30 μg protein were analysed by SDS–PAGE. MAP kinase was detected by chemiluminescence Western blotting method using specific MAP kinase antibody that recognizes the catalytically activated $p42^{mapk}$ and $p44^{mapk}$. (b) Following 24 h cultivation of confluent VSMCs in serum-free medium, cells were stimulated with 0.1 to 1.5% ethanol for 15 min. (c) Following 24 h cultivation of confluent VSMCs in serum-free medium, cells were preincubated with 20 μM PD98059 for 30 min before treatment with 1.0% ethanol for 15 min. (d) Confluent VSMCs were preincubated in serum-free medium for 24 h before stimulation with 50 ng ml^{-1} PDGF-BB for different time periods. (e) Following 24 h cultivation of confluent VSMCs in serum-free medium, cells were preincubated with 20 μM PD98059 for 30 min before stimulation with 50 ng ml^{-1} with PDGF-BB for 5 min. (a–e) show representative experiments from three independent experiments. (f and g) Densitometric analysis from data obtained from three separate experiments showing in (a, b and d). Data are expressed as a per cent of the phosphorylation of $p44^{mapk}/p42^{mapk}$ at 5 min by PDGF-BB and at 15 min by 1% ethanol (f) or as a per cent of the phosphorylation by 1.5% ethanol (g) (mean$\pm$s.e.mean, $n=3$, $^*P<0.05$ for PDGF-BB-stimulated versus unstimulated cells, $\S P<0.05$ for ethanol-treated versus untreated cells).

pase 3 enzymatically cleaves the ACF from the peptide and releases free ACF substrate that can be detected by measurement of the fluorescence at a wavelength of 530 nm using an excitation wavelength of 380 nm. Briefly, confluent VSMCs in 10-cm petri dishes were preincubated in 5 ml serum-free medium for 24 h before addition of 1.0% ethanol for different time periods. Cells were then scraped, centrifuged by 1500 r.p.m. for 5 min and washed three times with 5 ml PBS. Cells were then suspended in 150 μl apopain lysis buffer containing HEPES 10 mM, EDTA 2 mM, 0.1% 3-[3-Cholamidopropyl)dimethylammonio]-1 propanesulphonate (CHAPS), dithiothreitol (DTT) 5 mM, phenylmethylsulphoxide (PMSF) 1 mM, pepstatin A 15 μM, aprotinin 1.5 μM and leupeptin 43 μM, pH 7.4, and lysed by freezing and thawing of the samples by transferring from a methanol-dry ice bath to a 37°C water bath. The lysed cell extracts were transferred to microfuge tubes and cell debris were removed by centrifugation at 9000 r.p.m. for 30 min. Forty μl from the supernatant were used to determine caspase 3 activity in 1 ml reaction buffer containing piperazine-N,N′-bis(2-ethane sulphonic acid (PIPES) 1 mM, EDTA 2 mM, 0.1% CHAPS, DTT 5 mM and AFC 50 μM. Fluorescence was measured at 180 min.

Materials

Fura 2/AM and PD98059 were obtained from Calbiochem, Bad Soden, Germany. DMEM, Ham's F-10 and PBS were obtained from Gibco BRL (Eggestein, Germany). FCS was obtained from Boehringer Mannheim, Mannheim, Germany. PDGF-BB was a gift from Professor Dr Jürgen Hoppe, Physiological Chemistry, University of Würzburg, Germany and was prepared as described previously (Hoppe *et al*., 1989). [Methyl-^{3}H]-thymidine (74 GBq mol^{-1}) and myo-[2-^{3}H]-inositol (370 GBq mol^{-1}) were obtained from Amersham, Little Chalfont, England. Hybond N$^+$ membranes and ECL Western blotting detection system were obtained from Amersham, Little Chalfont, England. PhosphoPlus™ MAPK antibody Kit was obtained from New England BioLabs, Inc., Beverly, CA, U.S.A. Ethanol (p.a.) was obtained from Merck (Darmstadt, Germany). FluorAce™ apopain assay kit for fluorimetric detection of apoptosis was obtained from Bio-Rad (Munich, Germany).

Statistics

Values are expressed as the arithmetic mean ± s.e.mean. Statistical analysis of the data was performed using the Mann-Whitney *U*-test.

Results

As shown in Figure 1a, treatment of VSMCs with 0.3 to 1.5% ethanol for 24 h resulted in the complete abolition of [^{3}H]-thymidine incorporation into cell DNA, whereas stimulation by PDGF-BB resulted in a 3 fold increase. Ethanol at a concentration of 0.1% had no effect on DNA synthesis. The mean diameter of untreated intact VSMCs was 12.4 ± 0.16 μm (Figure 1b, control, representative measurement from three independent experiments). Treatment of the VSMCs with 1.0% ethanol led to the complete death of the VSMCs (Figure 1b, ethanol, no intact VSMCs were observed). In striking contrast, stimulation of the VSMCs with PDGF-BB (Figure 1b, PDGF-BB) resulted in an increase in cell count from $1.90 \times 10^6 \pm 41\,000$ to $2.37 \times 10^6 \pm 82\,000$ counts ml^{-1} (mean ± s.e.mean, $^*P < 0.05$ for PDGF-BB-treated versus untreated cells). The mean diameter of PDGF-BB-treated VSMCs was 13.8 ± 0.25 μm ($n = 3$, mean ± s.e.mean, $^*P < 0.05$ for PDGF-BB-treated versus untreated cells).

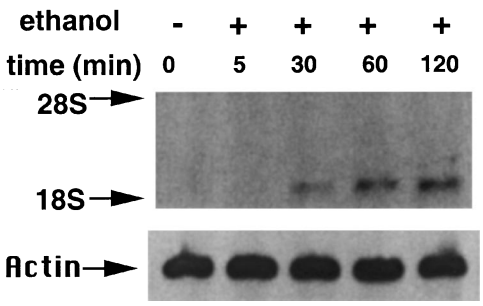

Figure 5 Effect of ethanol on induction of c-fos mRNA expression in VSMCs. Confluent cells in cm^2 flasks were precultured in serum-free medium for 24 h. Then cells were treated with 1.0% ethanol for different time periods. Ten μg of total RNA were electrophoresed on formaldehyde-agarose gels, blotted onto Hybond N$^+$ membranes and probed with a ^{32}P-labelled 1.0 kb v-fos fragment. The same blot previously hybridized with 1.0 kb v-fos was rehybridized with a 0.77 kb cDNA probe for β-actin mRNA. Arrows show the 28S (4.6 kb), the 18S ribosomal RNA (1.8 kb), the 2.2 kb c-fos mRNA and the 2.0 kb β-actin mRNA.

VSMCs were treated for 15, 30, 60, 120, 180 min and 24 h with 0.1% ethanol and fluorescence was measured at 180 min. As shown in Figure 2, caspase 3 activity was not altered in ethanol-treated cells whereas treatment of the cells with 100 nM staurosporin resulted in a 6 fold increase of the caspase 3 activity over the basal value. Staurosporine is a classic apoptotic trigger (Leist *et al*., 1997).

Furthermore, ethanol caused time-dependent changes in cell morphology as inspected by phase contrast microscopy which correlated with the toxic effects of ethanol observed within 24 h of ethanol treatment (Figure 3). After treatment of the cells with ethanol they appear more granular and 'blebby'.

Activation of p44^{mapk}/p42^{mapk} is accompanied by phosphorylation of the Tyr204 residue. When VSMCs were treated with 1.0% ethanol a marked time-dependent phosphorylation of p44^{mapk}/p42^{mapk} was observed with maximal stimulation at 15 min (Figure 4a). Phosphorylation was decreased to basal values after 30 min treatment. To address whether G_i proteins are involved in the signalling transduction pathway of ethanol, we examined the effect of ethanol on p44^{mapk}/p42^{mapk} phosphorylation in pertussis toxin (PTX) treated cells. As shown in Figure 4a, there was no difference between the phosphorylated level in PTX-treated and untreated cells. Treatment of the cells with ethanol (0.3 to 1.5%) for 15 min caused a dose-dependent activation of p44^{mapk}/p42^{mapk} with maximal stimulation at a concentration of 1.5% ethanol (Figure 4b). Ethanol at a concentration of 0.1% did not alter p44^{mapk}/p42^{mapk} phosphorylation. To elucidate whether activation of MAP kinase by ethanol occurs *via* the MAP kinase kinase (MEK) we investigated the effect of ethanol on phosphorylation of p44^{mapk}/p42^{mapk} isoforms in VSMCs in the presence and absence of the selective MEK inhibitor [2-2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one] (PD98059) (Dudley *et al*., 1995). As previously described an almost complete inhibition of the PDGF-BB-induced activation of MAP kinase activity in VSMCs was observed in the presence of 20 μM PD98059 (Bornfeldt *et al*., 1997). As shown in Figure 4c, treatment of VSMCs with 20 μM PD98059 did not inhibit ethanol-induced phosphorylation of p44^{mapk}/p42^{mapk}. PDGF-

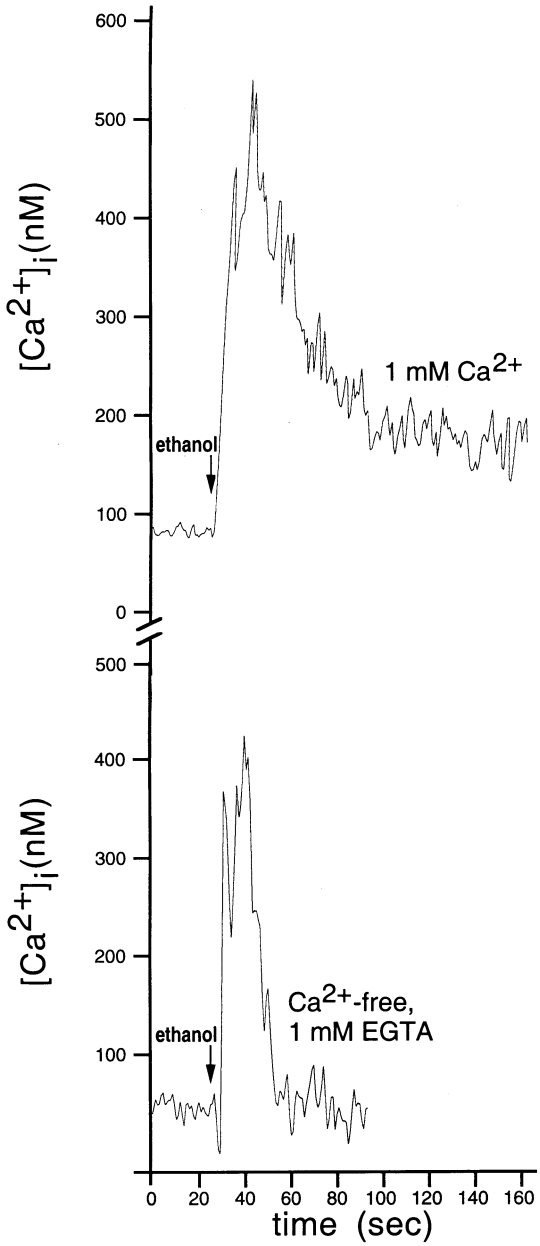

Figure 6 Effect of ethanol on $[Ca^{2+}]_i$ in VSMCs. Confluent VSMCs on slides were precultured for 24 h in serum-free medium. Then 1.0% ethanol was applied to fura-2-loaded VSMCs in the presence of 1 mM Ca^{2+} (HEPES buffer) and absence of extracellular Ca^{2+} (HEPES buffer without 1 mM Ca^{2+} containing 1 mM EGTA) and changes in fluorescence were monitored (representative curves from three separate independent experiments). After subtraction of autofluorescence, changes in 340/380 nm excitation wavelength ratio by the emission wavelength of 505 nm were converted into corresponding levels of $[Ca^{2+}]_i$.

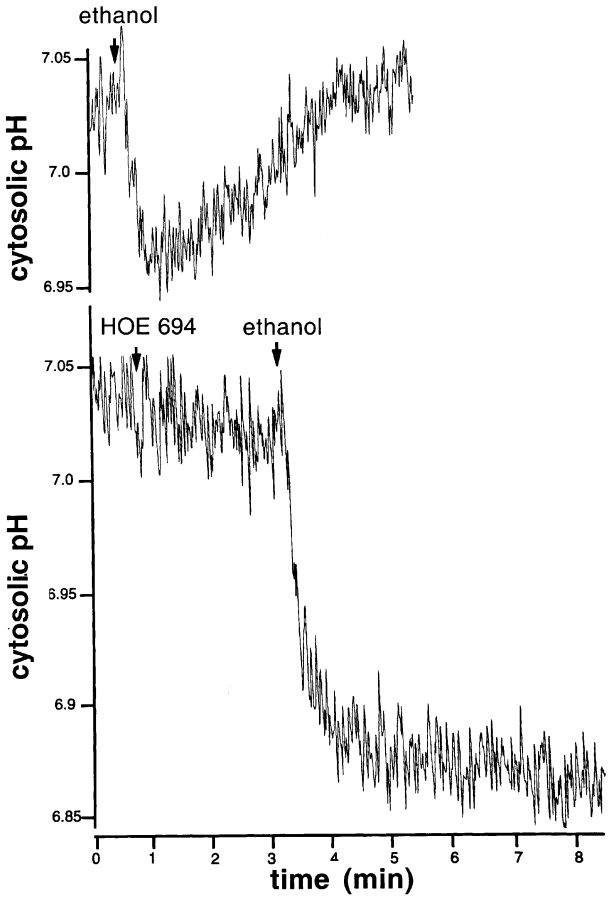

Figure 7 Effect of HOE 694 on the ethanol-induced intracellular pH_i changes. Confluent VSMCs on slides were precultured for 24 h in serum-free medium. *Upper curve*, Ethanol was applied to the BCECF-loaded cells in HEPES buffer. *Lower curve*, Cells were precultured with 10 μM HOE 694 for 2 min before addition of 1.0% ethanol. After calibration of the fluorescence signal by permeabilizing the cells with 1.3 μM nigericin changes in 492/438 nm excitation wavelength ratio by the emission wavelength 525 nm were converted into corresponding levels of pH_i (representative curves from three separate independent experiments).

BB caused a time-dependent increase of phosphorylation of $p44^{mapk}/p42^{mapk}$ with maximal phosphorylation at 5 min, which returned to the basal levels after 30 min (Figure 4d). Treatment of VSMCs with 20 μM PD98059 markedly inhibited PDGF-BB-induced activation of $p44^{mapk}/p42^{mapk}$ (Figure 4e). Quantification of the band densities by laser scanning densitometry obtained by three separate experiments show that maximal phosphorylation of the $p44^{mapk}/p42^{mapk}$ by PDGF-BB and ethanol occurred at 5 and 15 min, respectively (Figure 4f). Ethanol dose-dependently increases phosphorylation of the MAP kinase isoforms reaching a plateau at a concentration of 1.0%. A significant increase was observed at a concentration of 0.3% ethanol. Treatment of the VSMCs with 1.0% ethanol caused a time-dependent increase of the c-fos mRNA with maximal induction at 60 to 120 min (Figure 5).

In the absence of 1 mM extracellular Ca^{2+}, 1.0% ethanol induced an increase in $[Ca^{2+}]_i$ from 90 to 500 nM peaking at 10 s (Figure 6, upper curve, representative tracing from three experiments). After that $[Ca^{2+}]_i$ declined toward a stable value of 190 nM within 60 s. Stimulation of VSMCs in the absence of extracellular Ca^{2+} also resulted in an increase in $[Ca^{2+}]_i$ from 50 to 400 nM with a peak at 10 s (Figure 6, lower curve). Subsequently $[Ca^{2+}]_i$ declined to the basal value within 30 s.

Ethanol induced an initial rapid cytosolic acidification of 0.07 pH_i units that peaked at 30 s, which was followed by an alkalization toward baseline within 5 min (Figure 7, upper curve, representative tracing from three experiments). As we previously described, treatment of the cells with 10 μM HOE 694 completely inhibited the Ang II- and PDGF-BB-induced stimulation of the Na^+/H^+ exchanger in VSMCs (Sachinidis *et al.*, 1996a,b). Following incubation with HOE 694 (10 μM), a specific inhibitor of the Na^+/H^+ exchanger, ethanol elicited only intracellular acidification of 0.15 pH units below basal value (Figure 7, lower curve). Secondary alkalization to baseline, attributable to activation of the Na^+/H^+ exchanger, was not observed. Preliminary experiments demonstrated that

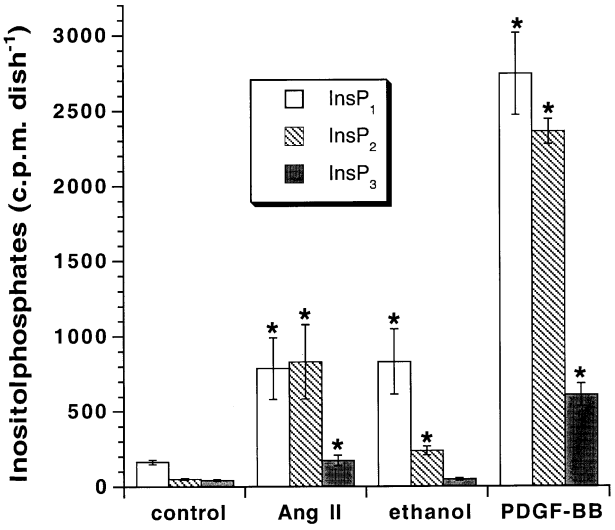

Figure 8 Effect of ethanol on the inositolphosphates formation in VSMCs. Cells were seeded in petri dishes (60 mm diameter) and grown in the presence of myo-[2-^{3}H]-inositol (4 μCi ml^{-1}) until confluence. Then cells were treated with 1.0% ethanol for 10 min or stimulated with 100 nM Ang II for 3 min and 50 ng ml^{-1} PDGF-BB for 10 min in a DMEM containing 20 mM LiCl. After extraction, analysis of inositolphosphates was performed in an anion exchanger Partisil SAX column by HPLC. Results are from one experiment performed in triplicate petri dishes and expressed as mean $\pm$ s.e.mean, $n=3$, $^*P<0.05$ for ethanol, Ang II, PDGF-BB effect versus control.

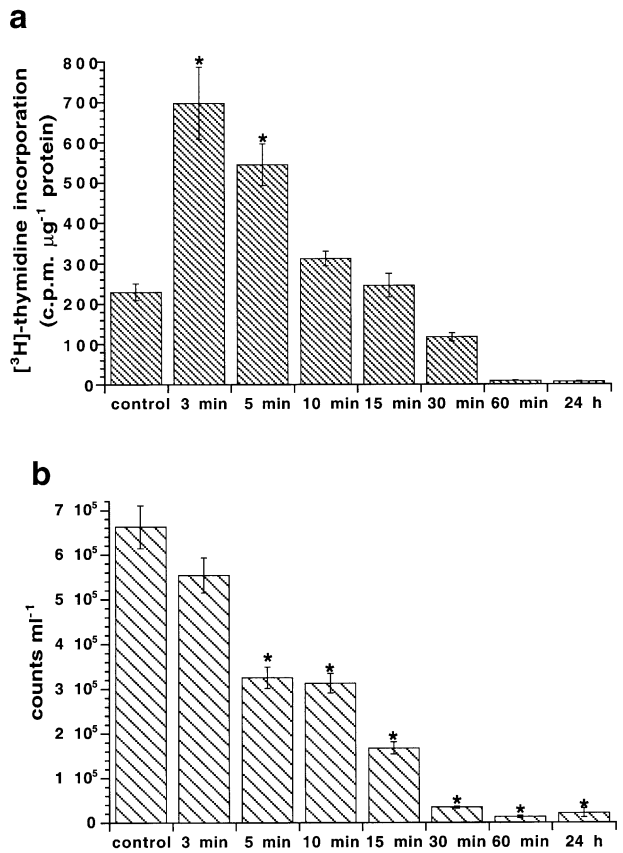

Figure 9 Effect of ethanol on [^{3}H]-thymidine incorporation into VSMCs DNA synthesis and cell counts after treatment of VSMCs with ethanol for different times. (a) Confluent VSMCs were precultured in 24-well plates for 24 h in serum-free medium. Then cells were treated with 1.0% ethanol for different time periods. The medium was then replaced with serum-free medium without ethanol. After 20 h of incubation, cells were exposed to 3 μCi ml^{-1} [^{3}H]-thymidine. Four hours later the reaction was terminated and [^{3}H]-thymidine incorporation into cell DNA and cell protein were quantified. (b) Confluent VSMCs were precultured in 24-well plates for 24 h of serum-free medium. Then cells were treated with 1.0% ethanol for different time periods. Then the medium was replaced with serum-free medium without ethanol. After 24 h the cells were trypsinized and intact cell counts were determined with the cell counter system CASY-1 (Schärfe System). Results are from a representative experiment from three independent experiments, performed in triplicate wells and are expressed as mean $\pm$ s.e.mean, $n=3$, $^*P<0.05$ for ethanol effect versus control.

treatment of the cells with 1.0% ethanol for 1, 3, 6, 10 and 20 min caused a time-dependent increase in $InsP_1$ and $InsP_2$ reaching maximal values at 10 min. However, no stimulation of $InsP_3$ production was obtained (data not shown). The ability of ethanol to stimulate intracellular formation of inositolphosphates was compared to that of PDGF-BB and Ang II. As previously described, maximal formation of inositolphosphates by 100 nM Ang II (Griendling *et al.*, 1986) and 50 ng ml^{-1} PDGF-BB (Sachinidis *et al.*, 1990) occurs at 3 and 10 min respectively. Therefore, we stimulated cells with either 10^{-7} M Ang II or 50 ng ml^{-1} PDGF-BB. Ang II induced a 3.8 fold, 15 fold and 3 fold increase in $Insp_1$, $InsP_2$, and $InsP_3$, respectively. PDGF-BB caused at 10 min a 16 fold, 45 fold and a 15 fold increase in $InsP_1$, $InsP_2$, and $InsP_3$, respectively, (Figure 8). Stimulation of the VSMCs with 1.0% ethanol for 10 min resulted in a 4 fold increase in both $InsP_1$ and $InsP_2$ formation, respectively. No formation of $InsP_3$ was observed.

These findings demonstrate that ethanol appears to have two separate effects on VSMCs. On the one hand treatment of VSMCs with ethanol for 24 h causes necrosis of VSMCs. On the other hand, ethanol stimulates early mitogenic events normally stimulated by classic growth factors. In order to distinguish the growth signalling pathway from its toxic effects, VSMCs were treated with ethanol for short times and then DNA synthesis and the cell number was determined. As demonstrated in Figure 9a, treatment of VSMCs with 1.0% ethanol for 3–5 min led to a remarkable increase in DNA synthesis. Treatment of the cells longer than 30 min completely inhibited DNA synthesis. Moreover, treatment of VSMCs with ethanol for 3 min had no significant effect on cell number (Figure 9b) while after treatment for 5–15 min only 50 or 25%, respectively, of intact VSMCs were obtained. Remarkably, almost no intact VSMCs were detected after their treatment with ethanol for longer than 30 min (Figure 9b).

Discussion

Early intracellular signalling events associated with PDGF-BB or Ang II stimulation of VSMCs are the stimulation of phosphatidylinositol bisphosphate (PIP_2) hydrolysis, release of $[Ca^{2+}]_i$ from intracellular stores, activation of the Na^+/H^+ exchanger, stimulation of the $p42^{mapk}/p44^{mapk}$ and stimulation of the c-fos mRNA expression. We found that, similar to growth factors, ethanol at concentrations of 0.3–1.5% is also able to stimulate these early events. In striking contrast to PDGF-BB and Ang II which are potent growth factors for VSMCs, treatment of the VSMCs with ethanol for longer than 30 min resulted in a complete VSMC death.

It has been established that the classical growth factor PDGF-BB propagates its mitogenic signals *via* autophosphorylation of the PDGF β-receptor tyrosine residues. This results in tyrosine phosphorylation of different substrate proteins such as phospholipase C-γ1 (PLC-γ1), $p21^{ras}$ GTPase activating protein (GAP) and phosphatidylinositol-(PI-3)

kinase all of which carry Src homology region 2 (SH_2)-domains capable of binding to regions of the PDGF β-receptor containing autophosphorylated tyrosine residues (Rönnstrand *et al*., 1992). Activation of $p44^{mapk}/p42^{mapk}$, is a key step in the growth signal transduction mediated through tyrosine kinase receptors such as the PDGD β-receptor. In this context, it has been postulated that activation of $p44^{mapk}/p42^{mapk}$ is involved in growth factor-induced expression of immediate-early genes such as c-fos (Blenis, 1993; Pelech & Sanghera, 1992). It has been demonstrated that activation of the $p44^{mapk}/p42^{mapk}$ occurs *via* MEK by phosphorylation of threonine and tyrosine residues and that MEK is activated by Raf-1 kinase (Blenis, 1993; Pelech & Sanghera, 1992).

The MAP kinase pathway can also be stimulated in response to stress factors like irradiation, DNA damaging chemicals, protein synthesis inhibitors, heat shock, osmotic imbalance (Fanger *et al*., 1997; Robinson & Cobb, 1997). We found that like PDGF-BB and Ang II, the stress factor, ethanol, was able to stimulate $p44^{mapk}/p42^{mapk}$.

Treatment of the cells with 20 μM PD98059 resulted in an 80% inhibition of the PDGF-BB-induced phosphorylation of $p44^{mapk}/p42^{mapk}$. In contrast to these findings, the MAP kinase phosphorylation by ethanol was not significantly attenuated by PD98059 suggesting that MAP kinase activation by ethanol is not mediated through MEK. Apparently, another kinase(s) is responsible for the phosphorylation of $p44^{mapk}/p42^{mapk}$. There is some evidence supporting our findings. (1) Recently, it has been shown that the contractile action of similar concentrations of ethanol was not affected by PD98059 whereas the contractile action of epidermal growth factor (EGF) was significantly inhibited by the MEK inhibitor (Zheng *et al*., 1997); (2) the bisperoxovanadium 1,10-phenanthroline-induced stimulation of MAP kinase in primary rat hepatocytes could not be abrogated by PD98059, suggesting that MAP kinases can also be stimulated in a MEK-independent manner; and (3) MEK-independent pathways causing prolonged activation of $p44^{mapk}/p42^{mapk}$ have been described, dependent on the activation of PI-3 kinase and conventional PKCs (Grammer & Blenis, 1997).

In concordance with the finding that ethanol stimulates $p44^{mapk}/p42^{mapk}$ we observed an expression of c-fos mRNA. It is well known that, following stimulation of VSMCs by either Ang II or PDGF-BB, maximal induction of c-fos mRNA occurs at 30 min and returns to basal values at 60 min (Sachinidis *et al*., 1993; Taubman *et al*., 1989). In contrast, the maximal induction of c-fos mRNA by ethanol was observed after 120 min of treatment. These findings suggest that the ethanol-induced intracellular mechanisms causing stimulation of MAP kinases and expression of c-fos mRNA are distinct from those induced by PDGF-BB and Ang II.

Elevated $[Ca^{2+}]_i$ and stimulation of the Na^+/H^+ exchanger by growth factors have both been implicated as early intracellular signals upstream to MAP kinase activation which may be involved in the regulation of cellular functions such as contraction and growth (Hepler & Gilman, 1992; Rozengurt, 1986). In this context, another interesting finding was that ethanol increases $[Ca^{2+}]_i$. Like Ang II and PDGF-BB, elevation in $[Ca^{2+}]_i$ in response to ethanol in the presence of extracellular Ca^{2+} consisted of an initial rapid $[Ca^{2+}]_i$ peak at 10–20 s (designated as the rapid phase) followed by a decline reaching a steady state (designated as the plateau phase). Similar to PDGF-BB and Ang II, ethanol caused only the rapid phase in the absence of extracellular Ca^{2+}. These findings suggest that the first rapid phase of $[Ca^{2+}]_i$ may be caused by phosphoinositide-specific phospholipase C metabolites *via* Ca^{2+} mobilization from intracellular stores and that the second phase may be due to a Ca^{2+}-influx from extracellular Ca^{2+}.

Using the specific Na^+/H^+ exchanger inhibitor HOE 694 we provide evidence that ethanol stimulates the Na^+/H^+ exchanger. Inhibition of the Ang II- and PDGF-BB-induced stimulation of the Na^+/H^+ exchanger by HOE 694 has already been shown (Sachinidis *et al*., 1996a,b). It is established that Ang II and PDGF-BB activate the phosphatidylinositol-specific phospholipase C (PLC)-β or PLC-γ1, respectively, resulting in an elevation of $InsP_3$ and diacylglycerol (DAG) (Griendling *et al*., 1986; Sachinidis *et al*., 1990). It is assumed that DAG stimulates protein kinase C (PKC) resulting in an activation of the Na^+/H^+ exchanger (Berridge & Irvine, 1989). Therefore, we examined whether plasma membrane phosphoinositol metabolites may be involved in the effects exerted by ethanol. We found that like Ang II and PDGF-BB, ethanol induced formation of $InsP_1$ and $InsP_2$. Also, like Ang II, ethanol caused a 4 fold increase in $InsP_1$. In comparison to Ang II that caused a 15 fold increase in $InsP_2$, ethanol induced only a 4 fold elevation of $InsP_2$. One noteworthy finding was that in contrast to PDGF-BB and Ang II, $InsP_3$ formation was not observed by ethanol. There are three possible explanations for this finding. (1) $InsP_3$ is rapidly hydrolyzed to $InsP_1$ and $InsP_2$; (2) $InsP_1$ and $InsP_2$ are derived from phosphatidylinositol phosphate (PIP_1); or (3) it is possible that ethanol may stimulate formation of $InsP_3$ earlier than 60 s which represents the first time point from our preliminary studies (data not shown). Since it is assumed that $InsP_3$ mobilizes Ca^{2+} from intracellular stores and ethanol stimulates elevation of $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} we favour the third hypothesis. In addition, we suggest that hydrolysis of PIP_2 by ethanol occurs *via* a direct stimulation of phospholipase C. These observations agree with the findings suggesting that ethanol stimulates a PLC-dependent elevation of $InsP_3$ and Ca^{2+} mobilization in rat hepatocytes (Hoek *et al*., 1990). The same group also suggest that activation of PLC occurred due to an interaction of ethanol with the intramembrane complex of receptor guanosine triphosphate (GTP) binding protein (G protein) and PLC, presumably causing the release of bound guanosine diphosphate (GDP) and the subsequent activation of G protein that controls the PLC activity (Hoek *et al*., 1990). In this context, it is established that the pertussis toxin-insensitive G_q subfamily can couple to several receptors and thereby modulate the specific PLC-β1 which catalyses the hydrolysis of PIP_2 with subsequent formation of $InsP_3$ and DAG. Since we demonstrated that PTX did not influence phosphorylation of $p44^{mapk}/p42^{mapk}$ we may suggest that in contrast to G_q proteins, PTX-sensitive G_i proteins do not play a decisive role in the early signalling events caused by ethanol. Furthermore, we found that treatment of VSMCs with ethanol for 3–5 min resulted in a marked increase in DNA synthesis without cell division. This finding suggests that the ethanol-induced early mitogenic signals promote only transition of the cells from the G0/G1 phase to the S phase of the cell cycle without progression to the M phase. In this context, it is well established that the early mitogenic signals determined in the present study are essential for progression from the G_0/G_i phase to the S phase of the cell cycle (Murray & Hunt, 1993). Treatment of the cells for longer than 30 min resulted in complete cell death. These findings suggest that stimulation of the early mitogenic signals including the stimulation of $p44^{mapk}/p42^{mapk}$ may represent a secondary stress-induced event by which the cells attempt to counteract the toxic effects of ethanol. Caspase 3 (apopain) is derived from the proenzyme CPP32 at the onset of apoptosis (Goldberg *et al*., 1996). Since ethanol did not influence the activity of caspase 3 we suggest

that ethanol promotes necrosis of VSMCs which might be mediated by a damage to plasma membranes.

We suppose that the activation of these signals reflects a cell effort to protect itself from a toxic substance that mediates cell death. Moreover, our results illustrate the need for a careful interpretation of all data regarding the antiproliferative effect of ethanol on different cell types including VSMCs (Hendrickson *et al*., 1998; Liu *et al*., 1997), hepatocytes (Chen *et al*., 1998; Tombes *et al*., 1998) and glia cells (Luo & Miller, 1997). Furthermore since ethanol at concentrations greater than 0.1% stimulates early intracellular events normally induced by growth factors, our results illustrate the need for a careful interpretation of all data obtained in biomedical research by the use of ethanol soluble agents. In order to avoid early intracellular events induced by ethanol its final concentration should be lower than 0.1% ethanol (17 mM).

References

ABEDI, H. & ZACHARY, I. (1995). Signalling mechanisms in the regulation of vascular cell migration. *Cardiovasc. Res.,* **30,** 544–556.

BERRIDGE, M.J., DAWSON, R.M.C., DOWNES, C.P., HESLOP, J.P. & IRVINE, R.F. (1983). Changes in the levels of inositolphosphates after agonist-dependent hydrolysis of membrane phosphoinositides. *Biochem. J.,* **212,** 473–482.

BERRIDGE, M.J. & IRVINE, R.F. (1989). Inositolphosphates and cell signalling. *Nature,* **341,** 197–205.

BLENIS, J. (1993). Signal transduction via the MAP kinases: Proceed at your own RSK. *Proc. Natl. Acad. Sci. U.S.A.,* **90,** 5889–5892.

BORNFELDT, K.E., CAMPBELL, J.S., KOYAMA, H., ARGAST, G.M., LESLIE, C.C., RAINES, E.W., KREBS, E.G. & ROSS, R. (1997). The mitogen-activated protein kinase pathway can mediate growth inhibition and proliferation in smooth muscle cells. Dependence on the availability of downstream targets. *J. Clin. Invest.,* **100,** 875–885.

BRADFORD, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.,* **72,** 248–254.

CHAMLEY, J.H., CAMPBELL, G.R. & ROSS, R. (1979). The smooth muscle cell in culture. *Physiol. Rev.,* **39,** 1–61.

CHEN, J., ISHAC, E.J., DENT, P., KUNOS, G. & GAO, B. (1998). Effects of ethanol on mitogen-activated protein kinase and stress-activated protein kinase cascades in normal and regenerating liver. *Biochem. J.,* **334,** 669–676.

CHO, A., MITCHELL, L., KOOPMANS, D. & LANGILLE, B.L. (1997). Effects of changes in blood flow rate on cell death and cell proliferation in carotid arteries of immature rabbits. *Circ. Res.,* **81,** 328–337.

CHOMCZYNSKI, P. & SACCHI, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.,* **162,** 156–159.

DAEMEN, M.J.A.P., LOMBARDI, D.M., BOSMAN, F.T. & SCHWARTZ, S.M. (1991). Angiotensin II induces smooth muscle cell proliferation in the normal and injured rat arterial wall. *Circ. Res.,* **68,** 450–456.

DUDLEY, D.T., PANG, L., DECKER, S.J., BRIDGES, A.J. & SALTIEL, A.R. (1995). A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc. Natl. Acad. Sci. U.S.A.,* **92,** 7686–7689.

DUFF, J.L., MARRERO, M.B., PAXTON, W.G., SCHIEFFER, B., BERNSTEIN, K.E. & BERK, B.C. (1995). Angiotensin II signal transduction and the mitogen-activated protein kinase pathway. *Cardiovasc. Res.,* **30,** 511–517.

DZAU, V.J. & GIBBONS, G.H. (1993). Vascular remodeling: mechanisms and implications. *J. Cardiovasc. Pharmacol.,* **21** (Suppl 1), S1–S5.

FANGER, G.R., GERWINS, P., WIDMANN, C., JARPE, M.B. & JOHNSON, G.L. (1997). MEKKs, GCKs, MLKs, PAKs, TAKs, and Tpls: upstream regulators of the c-Jun amino-terminal kinases. *Curr. Opin. Gen. Dev.,* **7,** 67–74.

GOLDBERG, Y.P., NICHOLSON, D.W., RASPER, D.M., KALCHMAN, M.A., KOIDE, H.B., GRAHAM, R.K., BROMM, M., KAZEMI-ESFARJANI, P., THORNBERRY, N.A., VAILLANCOURT, J.P. & HAYDEN, M.R. (1996). Cleavage of Huntington by apopain, a proapoptotic cysteine protease, is modulated by the polyglutamine tract. *Nat. Genet.,* **13,** 442–449.

GRAMMER, T.C. & BLENIS, J. (1997). Evidence of MEK-independent pathways regulating the prolonged activation of ERK-MAP kinases. *Oncogenes,* **14,** 1635–1642.

GRIENDLING, K.K., RITTENHOUSE, S.E., BROCK, T.A., EKSTEIN, L.S., GIMBRONE, M.A.J. & ALEXANDER, R.W. (1986). Sustained diacylglycerol formation from inositol phospholipids in angiotensin II-stimulated vascular smooth muscle cells. *J. Biol. Chem.,* **261,** 5901–5906.

GRYNKIEWICZ, G. POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.,* **260,** 3440–3450.

HAHN, G.M., SHIU, E.C. & AUGER, E.A. (1991). Mammalian stress protein HSP70 and HSP28 coinduced by nicotine and either ethanol or heat. *Mol. Biol. Cell,* **11,** 6034–6040.

HENDRICKSON, R.J., CAHILL, P.A., McKILLOP, I.H., SITZMANN, J.V. & REDMOND, E.M. (1998). Ethanol inhibits mitogen activated protein kinase activity and growth of vascular smooth muscle cells in vitro. *Eur. J. Pharmacol.,* **362,** 251–259.

HEPLER, J. & GILMAN, A.G. (1992). G proteins. *Trends Biochem. Sci.,* **17,** 383–387.

HOEK, J.B., TARASCHI, T.F., HIGASHI, K., RUBIN, E. & THOMAS, A.P. (1990). Phospholipase C activation by ethanol in rat hepatocytes is unaffected by chronic ethanol feeding. *Biochem. J.,* **272,** 59–64.

HOPPE, J., WEICH, H.A. & EICHNER, W. (1989). Preparation of biologically active PDGF type BB from a fusion protein expressed in Escherichia Coli. *Biochemistry,* **28,** 2956–2960.

LEIST, M., SINGLE, B., CASTOLDI, A.F., KUHNLE, S. & NICOTERA, P. (1997). Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. *J. Exp. Med.,* **185,** 1481–1486.

LIU, W.M., ANDERSON, P.G., LUO, J.F. & ROUBIN, G.S. (1997). Local delivery of ethanol inhibits intimal hyperplasis in pig coronary arteries after balloon injury. *Circulation,* **96,** 2295–2301.

LUO, J. & MILLER, M.W. (1997). Basic fibroblast growth factor- and platelet-derived growth factor-mediated cell proliferation in B104 neuroblastoma cells: effect of ethanol on cell cycle kinetics. *Brain Res.,* **770,** 139–150.

MARSHALL, C.J. (1995). Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell,* **80,** 179–185.

MILARSKY, K.L. & MORIMOTO, R.I. (1986). Expression of human HSP70 during the synthetic phase of the cell cycle. *Proc. Natl. Acad. Sci. U.S.A.,* **83,** 9517–9521.

MURRAY, A. & HUNT, T. (1993). Growth control in mammalian cells. In *The cell cycle: an introduction*. Oxford University Press, Inc: New York, New York. pp 100–116.

PELECH, S.L. & SANGHERA, S. (1992). MAP kinases: charting the regulatory pathways. *Science,* **257,** 1355–1356.

RINK, T.J., TSIEN, R.Y. & POZZAN, T. (1982). Cytoplasmic pH and free Mg^{2+} in lymphocytes. *J. Cell Biol.,* **95,** 189–196.

ROBINSON, M.J. & COBB, M.H. (1997). Mitogen-activated protein kinase pathways. *Curr. Opin. Cell Biol.,* **9,** 180–186.

RÖNNSTRAND, L., MORI, S., ARRIDSON, A.K., ERIKSSEN, A., WERNSTEDT, C., HELLMAN, U., CLAESSON-WELSH, L. & HELDIN, C.H. (1992). Identification of two C-terminal autophosphorylation sites in the PDGF-β-receptor: involvement in the interaction with phospholipase C-γ. *EMBO J.,* **11,** 3911–3919.

ROSS, R. (1993). The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature,* **362,** 801–809.

ROZENGURT, E. (1986). Early signals in the mitogenic response. *Science,* **234,** 161–166.

SACHINIDIS, A., FLESCH, M., KO, Y., SCHRÖR, K., BÖHM, M., DÜSING, R.. & VETTER, H. (1995). Thromboxane A2 and vascular smooth muscle cell proliferation. *Hypertension,* **26,** 771 – 780.

SACHINIDIS, A., LOCHER, R., HOPPE, J. & VETTER, W. (1990). The platelet-derived growth factor isomers, PDGF-AA, PDGF-AB and PDGF-BB, induce contraction of vascular smooth muscle cells by different intracellular mechanisms. *FEBS Lett.,* **275,** 95 – 98.

SACHINIDIS, A., SCHULTE, K., KO, Y., MEYER ZU BRICKWEDDE, M.K., HOPPE, V., HOPPE, J. & VETTER, H. (1993). The induction of early response genes in rat smooth muscle cells by PDGF-AA is not sufficient to stimulate DNA-synthesis. *FEBS Lett.,* **319,** 221 – 224.

SACHINIDIS, A., SEUL, C., KO, Y., DÜSING, R. & VETTER, H. (1996a). Effect of the Na^+/H^+ antiport inhibitor Hoe 694 on the angiotensin II-induced vascular smooth muscle cell growth. *Br. J. Pharmacol.,* **119,** 787 – 796.

SACHINIDIS, A., SEUL, C., SEEWALD, S., KO, Y. & VETTER, H. (1996b). Activation of Na^+/H^+ exchanger is not involved in the PDGF-BB-induced DNA-synthesis in vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.,* **227,** 468 – 472.

TAUBMAN, M.B., BERK, B.C, IZUMO, S., TSUDA, T., ALEXANDER, R.W. & NADAL-GINARD, B. (1989). Angiotensin II induces c-fos mRNA in aortic smooth muscle. *J. Biol. Chem.,* **264,** 526 – 530.

TIMMERMANS, P.B., WONG, P.C., CHIU, A.T., HERBLIN, W.F., BENFIELD, P., CARINI, D.J., LEE, R.J., WEXLER, R.R., SAYE, J.A. & SMITH, R.D. (1993). Angiotensin II receptors and angiotensin II receptor antagonists. *Pharmacol. Rev.,* **45,** 205 – 251.

TOMBES, R.M., AUER, K.L., MIKKELSEN, R., VALERIE, K., WYMANN, M.P., MARSHALL, C.J., MCMAHON, M. & DENT, P. (1998). The mitogen-activated protein (MAP) kinase cascade can either stimulate or inhibit DNA synthesis in primary cultures of rat hepatocytes depending upon whether its activation is acute/phasic or chronic. *Biochem. J.,* **330,** 1451 – 1460.

ZHENG, X.L., MOKASHI, S. & HOLLENBERG, M.D. (1997). Contractile action of ethanol in guinea pig gastric smooth muscle: inhibition by tyrosine kinase inhibitors and comparison with the contractile action of epidermal growth factor-urogastrone. *J. Pharmacol. Exp. Ther.,* **282,** 485 – 495.

(Received January 21, 1999
Revised September 27, 1999
Accepted September 29, 1999)