Protein Kinase C Activators Suppress Stimulation of Capillary Endothelial Cell Growth by Angiogenic Endothelial Mitogens

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Abstract. The intracellular events regulating endothelial cell proliferation and organization into formalized capillaries are not known. We report that the protein kinase C activator β-phorbol 12,13-dibutyrate (PDBu) suppresses bovine capillary endothelial (BCE) cell proliferation (K_{50} = 6 ± 4 nM) and DNA synthesis in response to human hepatoma-derived growth factor, an angiogenic endothelial mitogen. In contrast, PDBu has no effect on the proliferation of bovine aortic endothelial cells and is mitogenic for bovine aortic smooth muscle and BALB/c 3T3 cells. Several observations indicate that the inhibition of human hepatoma-derived growth factor–stimulated BCE cell growth by PDBu is mediated through protein kinase C. (*a*) Different phorbol compounds inhibit BCE cell growth according to their potencies as protein kinase C activators (12-O-tetradecanoylphorbol 13-acetate > PDBu >> β-phorbol 12,13-diacetate >>> β-phorbol; α-phorbol 12,13-dibutyrate; α-phorbol 12,13-didecanoate). (*b*) PDBu binds to a single class of specific, saturable sites on the BCE cell with an apparent K_d of 8 nM, in agreement with reported affinities of PDBu for protein kinase C in other systems. (*c*) Specific binding of PDBu to BCE cells is displaced by sn-1,2-dioctanoylglycerol, a protein kinase C activator and an analog of the putative second messenger activating this kinase in vivo. The weak protein kinase C activator, sn-1,2-dibutyrylglycerol, does not affect PDBu binding. (*d*) A cytosolic extract from BCE cells contains a calcium/phosphatidylserine–dependent protein kinase that is activated by sn-1,2-dioctanoylglycerol and PDBu, but not by β-phorbol. These findings indicate that protein kinase C activation can cause capillary endothelial cells to become desensitized to angiogenic endothelial mitogens. This intracellular regulatory mechanism might be invoked during certain phases of angiogenesis, for example when proliferating endothelial cells become differentiated to organize into nongrowing tubes.

ANGIOGENESIS, the formation of new capillaries, is relatively infrequent in normal adult tissues, but dramatically increases in growing tumors and several other pathological conditions (reviewed in Folkman, 1982). Angiogenesis in vivo requires not only increased proliferation of capillary endothelial cells but also organization of these cells into nonproliferating, differentiated blood vessels (Folkman et al., 1982). Endothelial cell proliferation is stimulated in vitro by two classes of heparin-binding growth factors that have been isolated from a variety of sources and are structurally related to fibroblast growth factor (Shing et al., 1984; Gospodarowicz et al., 1984; D'Amore and Klagsbrun, 1984; Maciag et al., 1984; Sullivan and Klagsbrun, 1985; reviewed in Lobb et al., 1986 and Thomas et al., 1986). Since many of these mitogens cause angiogenesis in the rat or rabbit cornea and the chick embryo (D'Amore, 1982; Shing et al., 1985; Klagsburn et al., 1986), it has been postulated that they are endogenous angiogenic factors. While capillary endothelial cell growth control is clearly a very important component of in vivo angiogenesis, the intracellular events that regulate endothelial cell response to angiogenic mitogens are not known.

In the present study, we have investigated the role of the phospholipid/calcium–dependent protein kinase (protein kinase C) in capillary endothelial cell growth. In many other cell types, protein kinase C is thought to be involved in mitogenesis. Several agents, including mitogens such as platelet-derived growth factor and bombesin, cause increased hydrolysis of phosphatidylinositol-4,5-bisphosphate in their target cells (Berridge and Irvine, 1984; Berridge, 1984; Michell, 1982; Berridge et al., 1984). One of the putative second messengers released upon phosphatidylinositol-4,5-bisphosphate hydrolysis is sn-1,2-diacylglycerol, which activates protein kinase C by increasing its affinity for calcium (Nishizuka, 1984). Certain phorbol diesters are potent activators of protein kinase C, binding at the same site as diacylglycerol (Castagna et al., 1982), and, it has been suggested that most, if not all, of the biological effects of these compounds are due to protein kinase C activation (Nishizuka, 1984; Kikkawa et al., 1983). One of these actions is the stimulation of cell division in such cell types as Swiss 3T3 cells (Rozengurt et al., 1984; Dicker and Rosengurt, 1980), lymphocytes (Kaibuchi et al., 1985; Gelfand et al., 1985), thyroid cells (Bachrach et al., 1985), and chick heart mesen-

chymal cells (Balk et al., 1984). Often, phorbol diesters exert their stimulatory effect in synergy with suboptimal concentrations of serum or other mitogens.

We have found that β-phorbol 12,13-dibutyrate (PDBu)[1] and other protein kinase C activators inhibit, rather than stimulate, the proliferation of bovine capillary endothelial (BCE) cells in response to angiogenic endothelial mitogens such as human hepatoma-derived growth factor (HDGF; Klagsbrun et al., 1986). In addition, we present evidence that the PDBu receptor in BCE cells shares several characteristics, including diacylglycerol specificity, with protein kinase C. These findings imply that this protein kinase may be involved in an intracellular regulatory mechanism to suppress the response of endothelial cells to angiogenic mitogens. We suggest that such a mechanism might be invoked during endothelial cell differentiation into nonproliferating capillary tubes.

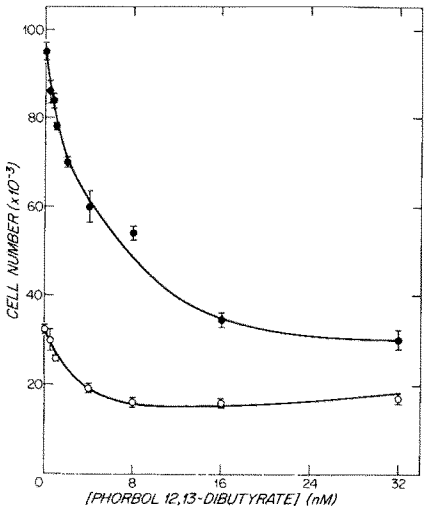

Figure 1. Inhibition of capillary endothelial cell proliferation by phorbol dibutyrate. The BCE cell proliferation assay was performed as described in Materials and Methods, with 15,000 cells plated per well. The number of attached cells per well when test substances were added was 14,100 ± 600 (n = 8). Cells were incubated with test substances for 72 h before cell counts were determined. Assays were performed with (*solid circles*) or without (*open circles*) 10 U/well of human HDGF (Klagsbrun et al., 1986). The results given are the means ± SEM of quadruplicate wells.

Materials and Methods

Materials

Tris base, Hepes, EGTA, EDTA, *p*-toluenesulfonylfluoride, aprotinin, BSA, and histone (type IIIS) were purchased from Sigma Chemical Co. (St. Louis, MO). Phorbol compounds were from Sigma Chemical Co. or from LC Services Corporation (Woburn, MA). The sodium salt of ATP was from P-L Biochemicals (Piscataway, NJ). [γ-^{32}P]ATP, [20-^{3}H(N)]phorbol 12,13-dibutyrate, [^{3}H-methyl]thymidine, [2-^{3}H(N)]myo-inositol, [5,6,9,11,12,14,15-^{3}H]arachidonic acid, and [^{3}H]toluene were from Dupont-New England Nuclear (Boston, MA). SDS and 2-mercaptoethanol were from Bio-Rad Laboratories (Richmond, CA). Thin-layer chromatography plates were from EM Reagents (Cincinnati, OH). Tissue culture reagents were from Gibco (Grand Island, NY). Highly purified human HDGF was prepared by Robert Sullivan and Dr. Michael Klagsbrun, Departments of Surgery and Biological Chemistry, The Children's Hospital and Harvard Medical School (Klagsbrun et al., 1986). The specific activity of this factor was 5 U/ng, with 1 U eliciting half-maximal stimulation of 3T3 cell DNA synthesis as described by Klagsbrun and Shing (1985). sn-1,2-Dioctanoylglycerol (DiC_8) and phosphatidylserine (PS) were obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). sn-1,2-Dibutyrylglycerol (DiC_4) was synthesized enzymatically and purified as described by Ebeling et al. (1985), using dibutyrylphosphatidylcholine from Avanti Polar Lipids, Inc., and phospholipase C (type XIII) from Sigma Chemical Co. The concentrations of diacylglycerol or phorbol diester in stock solutions were measured by an ester determination procedure using alkaline hydroxylamine and ferric perchlorate (Kates, 1972). All other reagents were from Fisher Scientific Co. (Pittsburgh, PA).

Cell Culture

BCE cells were isolated from bovine adrenal glands and cultured as described by Folkman et al. (1979), except that 5 μl/ml retinal extract (Gitlin and D'Amore, 1983; D'Amore et al., 1981) was substituted for the mouse sarcoma-conditioned medium. Bovine aortic endothelial cells and bovine aortic smooth muscle cells were isolated and grown as described previously (Folkman et al., 1979; Voyta et al., 1984). BALB/c 3T3 cells were obtained from Dr. Michael Klagsbrun.

Cell Proliferation Assays

Cell proliferation assays were performed in gelatinized 24-well dishes (Nunc, Roskilde, Denmark) as previously described (Klagsbrun and Shing, 1985; Shing et al., 1984). Unless otherwise specified, the medium was DME with 10% calf serum. PDBu or other phorbol compounds were added as solutions in dimethylsulfoxide (Me_2SO). Me_2SO was added to all test solutions and controls so that the final concentration was identical in all wells (<1%). After incubation with test substances for the indicated times, cells were trypsinized and counted using a cell counter (Coulter Electronics, Hialeah, FL). All assays were performed in quadruplicate.

Reversibility of PDBu Treatment

Cells were plated (50,000 cells/well) on a gelatinized 6-well dish (35-mm diameter wells) and were incubated overnight in DME-10% calf serum. The following day, the medium was changed to DME-10% calf serum containing either 0.001% Me_2SO (three wells) or 20 nM PDBu, 0.001% Me_2SO (three wells). The cells were incubated for 72 h, then the medium was replaced with DME-10% calf serum for 48 h. The cells from each experimental condition were trypsinized, pooled, and counted. Complete detachment of both types of cells was confirmed by light microscopy. The total cell number was 5.5×10^5 for the control and 1.3×10^5 for the PDBu-treated cultures. Cells were plated on gelatinized 24-well dishes at 10,000 cells/well (12 wells per condition) and were incubated in BCE growth medium. The medium was changed after 24 h and every 48 h thereafter. At each time point (1, 3, 5, and 7 d after plating) the cell numbers in control and PDBu-treated wells were determined. To compare growth rates, the logarithms of cell numbers (determined in triplicate wells) were plotted versus time (in days).

DNA Synthesis Assays

For experiments measuring the incorporation of [^{3}H]thymidine into acid-precipitable material, cells were plated in gelatinized 24-well dishes (16-mm wells) at 15,000 cells/well. For autoradiography, cells were plated at equivalent densities on gelatinized 35-mm dishes or tissue culture chamber/slides (LabTek; Miles, Laboratories, Elkhart, IN). Unless otherwise indicated, the medium was DME with 1% calf serum and 0.1% Me_2SO. Cells were incubated with [^{3}H-methyl]thymidine and other test substances as described in the figure legends. For determining acid-precipitable radioactivity, the medium was aspirated and the cells were washed with Dulbecco's PBS. The cells were then washed twice for 5 min with cold acetic acid/methanol (1:3) and twice for 10 min with cold 10% TCA. The precipitates were then washed with water and dissolved in three successive 0.15-ml aliquots of 0.3 N NaOH. For each well, these aliquots were pooled and added to 4 ml scintillation fluid (ReadySolv MP; Beckman Instruments, Inc., Fullerton, CA) containing 20 μl glacial acetic acid. Samples were counted for tritium in a scintillation spectrometer (LS 3801; Beckman Instruments, Inc.) and data were corrected for quenching using the H-number calibration

1. *Abbreviations used in this paper*: BCE, bovine adrenal capillary endothelial; DiC_4, sn-1,2-dibutyrylglycerol; DiC_8, sn-1,2-dioctanoylglycerol; HDGF, human hepatoma-derived growth factor; Me_2SO, dimethylsulfoxide; PDBu, β-phorbol 12,13-dibutyrate; PIP_2, phospatidylinositol-4,5-bisphosphate; PS, phosphatidylserine.

method with tritiated toluene as standard. For autoradiography, cells were washed with PBS and fixed successively in Karnovsky's fixative and 1% OsO_4. Slides or dishes were washed in water and allowed to air dry. Fixed cell monolayers were coated with Kodak Nuclear Track Emulsion, type NTB. After at least 1 wk exposure, the emulsions were developed and fixed according to the manufacturer's instructions.

PDBu Binding to BCE Cells

Cells were plated in gelatinized 24-well plates (16-mm diameter wells) at 15,000 cells/well and were incubated for 3–4 d in DME–10% calf serum. Immediately before binding assays were conducted, the cells from two or three wells were trypsinized and the average cell number was determined. The medium was aspirated from the remaining wells and the cells were washed with 1 ml PBS. The PBS was removed and replaced with [20-^{3}H(N)]phorbol 12,13-dibutyrate ([^{3}H]PDBu) in 0.3 ml DME with 0.1% BSA and 25 mM Hepes, pH 7.4. For concentrations >30 nM, nonradioactive PDBu was added as a carrier. For each concentration of PDBu tested, nonsaturable binding was determined in the presence of 20 μM unlabeled PDBu. Nonradioactive PDBu, when present, was added as a solution in Me_2SO and [^{3}H]PDBu was added as a solution in ethanol. (The radiochemical purity of [^{3}H]PDBu was confirmed by thin-layer chromatography on silica plates developed in toluene/acetone [3:1] followed by autoradiography.) Me_2SO and ethanol were added to all test solutions so that the final concentrations were 1 and 0.8%, respectively. The cells were incubated at 37°C for the indicated times, the medium was aspirated, and the cells washed three times with 1 ml cold 0.9% NaCl, 50 mM Tris-HCl, pH 7.4. The cells were solubilized in three successive aliquots of 0.15 ml 1% SDS. For each well, these aliquots were pooled and counted in 15 ml of ReadySolv MP scintillation fluid. Data were corrected for quenching as described above. Maximal binding of PDBu under these conditions occurred within 60 min and remained stable for at least 180 min.

Preparation of BCE Cytosol

All procedures were carried out at 4°C. Cells from two confluent 75-cm^2 flasks were detached by incubation with agitation in PBS with 0.5 mM EDTA for 20 min, followed by gentle scraping with a cell scraper (Costar, Cambridge, MA). The cells (1.5×10^7 cells) were collected by centrifugation and were resuspended in 0.5 ml of extraction buffer (50 mM Tris-HCl, pH 7.5, 0.5 mM EGTA, 2 mM EDTA, 1 mM *p*-toluenesulfonylfluoride, 20 mM 2-mercaptoethanol, and 10 μg/ml aprotinin). The mixture was homogenized in a 1-ml Dounce micro tissue grinder (Reactiware; Pierce Chemical Co., Rockford, IL), using five strokes with the A pestle. The tissue grinder was washed with an additional 1 ml extraction buffer, and the combined homogenate was centrifuged at 1,000 *g* for 10 min. The supernatant was set aside, and the pellet was resuspended in 1 ml extraction buffer and centrifuged in the same manner as above. The pooled 1,000-*g* supernatants (3-ml volume) were centrifuged at 100,000 *g* for 30 min. The resulting cytosolic supernatant was desalted by two successive cycles of concentration (to ~50–100 μl) and dilution (to 2 ml with extraction buffer minus aprotinin) in a (10-kD mol wt cutoff) microconcentration apparatus (Centricon 10; Amicon, Danvers, MA). The concentration steps were performed by centrifugation at 3,000 *g* for 2–4 h. The final cytosolic protein solution was collected and the membrane washed with extraction buffer, yielding a final volume of 400 μl. The protein concentration was determined with the Bio-Rad protein assay (Bio-Rad Laboratories), with BSA as a standard.

Protein Kinase Assays

Cytosolic preparations from BCE cells were incubated at 35°C in a reaction mixture containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.5 mg/ml histone (type IIIS; Sigma Chemical Co.), 50 μM [γ-^{32}P]ATP, and other substances as indicated. PS and DiC_8 were sonicated in water before addition to reactions. Reactions were terminated by spotting aliquots onto phosphocellulose filter paper (P81; Whatman, Inc., Clifton, NJ) and immediately washing with 75 mM phosphoric acid. The spotting and filtration were done in a Minifold apparatus (Schleicher and Schuell, Inc., Keene, NH) under vacuum. The filter paper was then removed from the apparatus and washed in 75 mM phosphoric acid. Spots were cut out from the filter paper and counted for ^{32}P in 4 ml ReadySolv EP scintillation fluid. When desired, the amount of [γ-^{32}P]ATP remaining in the reaction mixtures was determined by thin-layer chromatography as described previously (Doctrow and Lowenstein, 1985).

Measurement of Inositol Phosphate Production by BCE Cells

BCE cells (200,000 cells/well) were plated in DME–10% calf serum in 35-mm gelatinized tissue culture wells. After cells were attached, the medium was replaced with 1.5 ml DME–10% calf serum containing 10–25 μCi/ml [2-^{3}H(N)]-myo-inositol (^{3}H-inositol). (In later experiments, the medium was 10% DME–90% inositol-free DME [GIBCO special order] with 10% calf serum.) After prelabeling for 48 h, the cells were incubated for 30 min in PBS with 15 mM $LiCl_2$ (Besterman et al., 1986). The medium was then replaced with 1 ml PBS with or without HDGF (20 U). At this time, zero time samples were prepared by removing medium from some wells and adding 1 ml extraction solution (chloroform/methanol/HCl; 1:2:0.05). For each condition, duplicate or triplicate wells were prepared. The experimental wells were incubated for 20 min, then quenched with extraction solution as described for the zero time samples. All dishes were extracted for 20 min at room temperature, then washed twice with 1 ml extraction solution. Phase separation was achieved by adding 800 μl chloroform and 800 μl 1.2 N HCl. The aqueous phase was removed and the organic phase washed twice as described previously (Doctrow and Lowenstein, 1985). The washes were pooled with the aqueous phase and the solvent was removed in a Speed-Vac (Savant Instruments, Inc., Hicksville, NY) without heat. The water-soluble inositol phosphates were separated by ion exchange chromatography and the peaks corresponding to inositol monophosphate, inositol diphosphate, and inositol triphosphate were collected and counted as described previously (Berridge, 1983). To assess incorporation of [^{3}H]-inositol into phosphatidylinositol, phosphatidylinositol-4-phosphate, and phosphatidylinositol-4,5-bisphosphate (PIP_2), the organic layers were dried down and subjected to thin-layer chromatography in system III (Doctrow and Lowenstein, 1985) followed by autofluorography.

Results

PDBu inhibited the proliferation of BCE cells in a concentration-dependent manner (Fig. 1). In the absence of PDBu, the cells grew very slowly in serum alone, in agreement with previous reports (Folkman et al., 1979), while HDGF caused their growth rate to nearly triple. Even in the presence of HDGF, however, PDBu potently inhibited BCE cell growth. The concentration of the phorbol ester required to inhibit the number of population doublings by 50% was 6 ± 4 nM (four separate proliferation experiments). Cells were examined daily by phase-contrast microscopy and there was no indication that PDBu caused increased cell detachment. Allowing BCE cells to attach to culture dishes for 6 h in the presence of 200 nM PDBu did not affect cell plating efficiency. PDBu-treated cells and untreated cells stained equally well with the viability indicator fluorescein diacetate. In addition, PDBu-mediated growth inhibition was reversible. When BCE cells that had been incubated for 72 h in PDBu were removed from the phorbol ester and replated as described in Materials and Methods, PDBu-treated and control cells replated with similar efficiencies and grew at identical rates (data not shown).

To examine cell specificity, we compared the effect of PDBu on BCE cell proliferation with its effect on that of two other vascular cell types, bovine aortic endothelial cells, and bovine aortic smooth muscle cells. In contrast to its potent inhibition of BCE cells (Fig. 2 A), PDBu did not affect the proliferation of aortic endothelial cells (Fig. 2 B) even at 1 μM. The proliferation of aortic smooth muscle cells was also not inhibited by PDBu (Fig. 2 C), but was, in fact, slightly stimulated.

In shorter term assays for DNA synthesis, PDBu was also effective in inhibiting the response to HDGF. Fig. 3 shows that PDBu inhibited the incorporation of radioactive thymidine by BCE cells in response to increasing concentrations of HDGF. Inhibition by even suboptimal (15 nM) PDBu was

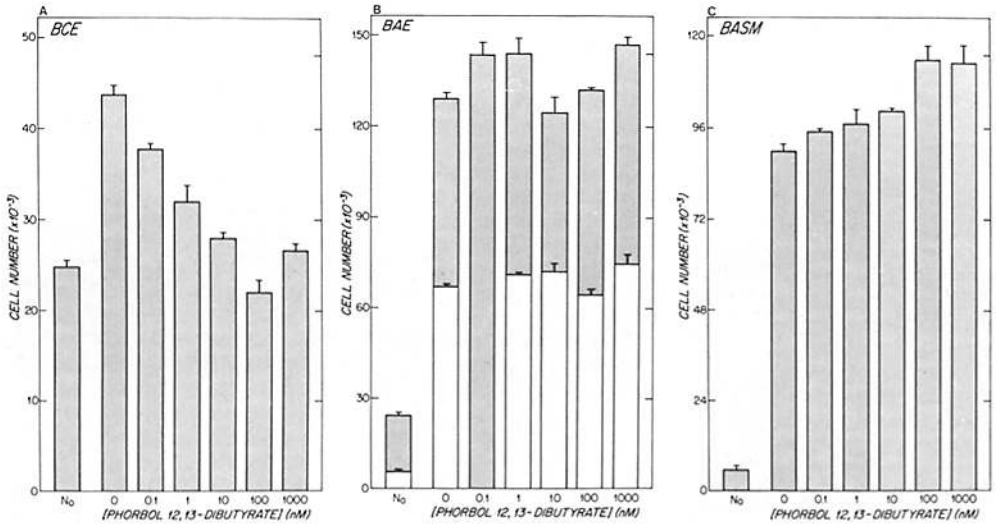

Figure 2. Effects of phorbol dibutyrate on proliferation of vascular cells. Cell proliferation assays were performed as described in Materials and Methods. Results are the means ± SEM of quadruplicate wells. N_0 is the number of cells attached per well at the beginning of the experiment. (*A*) BCE cells, plated at 30,000 cells/well were incubated for 72 h with test substances. (*B*) Bovine aortic endothelial cells, plated at 30,000 cells/well (*stippled bar*) and 7000 cells/well (*open bar*), and incubated for 48 h with test substances. (*C*) Bovine aortic smooth muscle cells, plated at 7,000 cells/well and incubated for 72 h with test substances.

not overcome by high levels (20 U/ml) of HDGF. Autoradiographic analysis confirmed the inhibition of HDGF action by PDBu. In the absence of factor, cells showed a basal DNA synthetic level corresponding to <20% labeled nuclei (Fig. 4). The percent of cells undergoing DNA synthesis was increased over threefold in the presence of HDGF. Addition of PDBu essentially eliminated the HDGF-stimulated DNA synthesis with no apparent effect on the basal level. In the presence or absence of HDGF, cells treated with PDBu displayed a morphological change, extending long processes (not shown).

While phorbol esters have been reported to increase cell growth in synergy with other mitogens, in this case PDBu and HDGF showed antagonistic rather than synergistic actions. This does not seem to be true in all cases of growth stimulation by this class of mitogen. In quiescent BALB/c 3T3 fibroblasts, DNA synthesis was stimulated by both PDBu and HDGF (Table I). Kaibuchi et al. (1985) recently showed that the stimulation of Swiss 3T3 cell growth by fibroblast growth factor (a growth factor structurally related if not identical to HDGF; Lobb et al., 1986) may involve activation of protein kinase C.

Since the inhibition of growth factor action is not a typical effect of protein kinase C activators, we further investigated the phorbol diester–induced inhibition of BCE cell proliferation for evidence that it was due to protein kinase C activation. We tested five different phorbol derivatives to determine whether there was a correlation between protein kinase C activators and inhibitors of BCE cell response to HDGF (Table II). The potent protein kinase C activator 12-O-tetradeca-

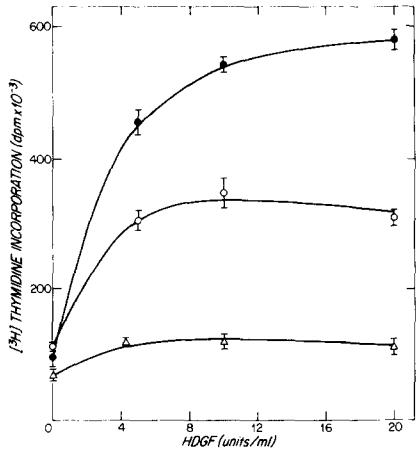

Figure 3. Effect of phorbol dibutyrate on the concentration-dependent response of capillary endothelial cells to HDGF. BCE cells were incubated for 24 h in medium containing 2 μCi/ml [^{3}H-methyl]thymidine and HDGF and PDBu as indicated. Incorporation of radioactivity into acid-precipitable material was determined as described in Materials and Methods. Each value represents the mean ± SEM of triplicate wells. The concentrations of PDBu were (*solid circles*) 0 nM; (*open circles*) 15 nM; and (*open triangles*) 150 nM.

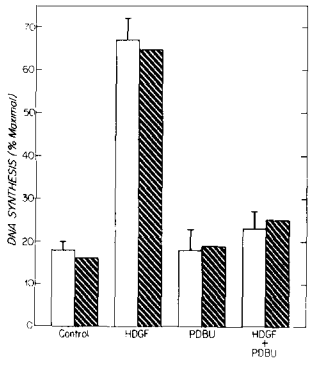

Figure 4. Phorbol dibutyrate-mediated suppression of growth factor–stimulated DNA synthesis by capillary endothelial cells. BCE cells were incubated for 18 h in medium containing, where indicated, 20 U/ml HDGF and 150 nM PDBu. Cells were labeled for an additional 3 h with [^{3}H-methyl] thymidine (2 μCi/ml) before processing for either autoradiography (*open bars*) or quantitation of acid-precipitable radioactivity (*striped bars*) as described in Materials and Methods. Autoradiography results are expressed as percent labeled nuclei with each value representing the mean ± SEM for at least five fields containing 35–85 cells. The acid-precipitable radioactivity per cell is expressed as percent of a maximal value arbitrarily defined as 50 dpm tritium incorporated per cell. Cell numbers were determined in parallel wells by Coulter counting.

Table I. Stimulation of BALB/c 3T3 Cell DNA Synthesis by PDBu or HDGF

Treatment	[^{3}H]Thymidine incorporation
	dpm
None	949 ± 74
PDBu	59,288 ± 1,733
HDGF	33,198 ± 1,084
PDBu + HDGF	111,186 ± 6,350

Incorporation of [^{3}H]thymidine by quiescent BALB/c 3T3 cells was determined as described previously (Klagsbrun and Shing, 1985). The wells contained 0.1% Me_2SO and, where indicated, 30 nM PDBu and 4 U per ml HDGF. Each value represents the mean ± SEM for quadruplicate wells.

noylphorbol 13-acetate (TPA) inhibited BCE cell proliferation slightly more than PDBu, with both causing >75% suppression at 100 nM. β-Phorbol and α-phorbol 12,13-didecanoate, which do not activate protein kinase C (Castagna et al., 1982), had no significant effect on BCE cell proliferation at 100 nM. At the same concentration, β-phorbol 12,13-diacetate, a weak protein kinase C activator (Noguchi et al., 1985), only slightly inhibited BCE cell proliferation. Similar relative potencies were observed for all compounds at 10 nM (data not shown). Another compound tested was α-phorbol 12,13-dibutyrate, an inactive stereoisomer of PDBu and therefore perhaps the most suitable control compound. This PDBu isomer had no effect on HDGF-stimulated proliferation or DNA synthesis at concentrations as high as 2 μM (data not shown).

We next investigated binding of PDBu to BCE cells using a radioactive ligand-binding assay. BCE cells exhibited specific, saturable binding of PDBu in the same concentration range as that causing inhibition of proliferation (Fig. 5). Scatchard analysis of these data revealed that PDBu bound at a single class of sites with an apparent K_d of ~8 nM. The maximal binding of PDBu was ~85 fmol/10^5 cells, or ~5 × 10^5 receptors per cell.

As noted above, phorbol diesters bind protein kinase C at the same site as the enzyme's presumed endogenous activator, sn-1,2-diacylglycerol (Nishizuka, 1984; Kikkawa et al., 1983). Recently, a series of synthetic diacylglycerols have been evaluated as activators of protein kinase C. The most potent of these is DiC_8, which activates isolated protein kinase C, displaces PDBu from its receptor, and evokes a number of cellular responses that have been attributed to protein kinase C activation (Ganong and Bell, 1985; Ebeling et al., 1985; Davis et al., 1985; Lapetina et al., 1985). Diacylglycerols with shorter chain lengths are much weaker. In par-

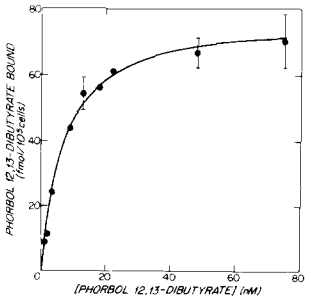

Figure 5. [^{3}H]PDBu binding to BCE cells. Binding was determined as described in Materials and Methods, with 33,000 cells/well and an incubation time of 120 min. The results shown are the mean ± SEM of triplicate determinations or the mean of duplicate determinations that agreed to within 5%. Nonsaturable binding of [^{3}H]PDBu, also determined in triplicate, has been subtracted from each value. Nonsaturable binding was 28 ± 1 fmol/10^5 cells at 13 nM [^{3}H]PDBu and 104 ± 12 fmol/10^5 cells at 48 nM [^{3}H]PDBu.

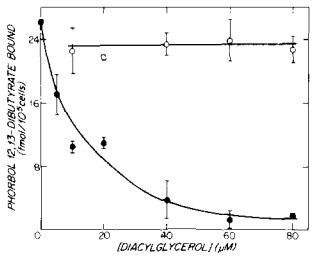

Figure 6. Inhibition of [^{3}H]-PDBu binding to capillary endothelial cells by sn-1,2-diacylglycerols. Binding of [^{3}H]-PDBu to BCE cells was measured as described in Materials and Methods with 39,000 cells/well, 10 nM [^{3}H]PDBu, and an incubation time of 60 min. Diacylglycerols in Me_2SO were added to the binding medium and the mixtures were sonicated before further dilution. Nonsaturable binding of [^{3}H]PDBu, determined in triplicate, was subtracted from each value. Shown is the binding of [^{3}H]PDBu to BCE cells in the presence of DiC_8 (*solid circles*) and DiC_4 (*open circles*). Each value is the mean ± SEM of three determinations.

Table II. Comparison of Effects of Different Phorbol Compounds on Protein Kinase C Activity and HDGF-induced Capillary Endothelial Cell Proliferation

Phorbol compound	Cell No.	Percent inhibition*	Percent protein kinase C activation	
			(Noguchi et al., 1985)	(Castagna et al., 1982)
None (control)	78,900 ± 1,600	–	–	–
β-Phorbol 12, 13-dibutyrate	19,700 ± 800	77	75	88
12-O-Tetradecanoylphorbol 13-acetate (β)	14,100 ± 400	96	95	100
β-Phorbol	76,900 ± 3,500	1	0	0
α-Phorbol 12, 13-didecanoate	71,600 ± 3,100	4	–	0
β-Phorbol 12,13-diacetate	56,600 ± 3,400	18	25	–

BCE cell proliferation assays were performed as described in Materials and Methods, with 17 U HDGF per well and 100 nM phorbol compound. The number of attached cells per well when test substances were added was 13,000. Cell numbers are the means ± SEM of quadruplicate wells. Previously published in vitro protein kinase C activation data were determined by Noguchi et al. (1985) at 20 nM and Castagna et al. (1982) at 10 ng/ml.
* Inhibition = 1 − (number of cell doublings with phorbol compound)/(number of cell doublings in control).
Number of cell doublings = $\log_2$ (cell number at 72 h)/(initial cell number).

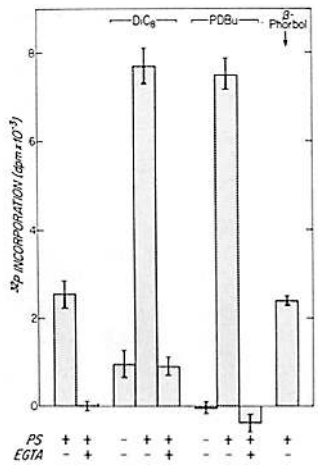

Figure 7. Activation of BCE cytosolic protein kinase C by DiC_8 or phorbol dibutyrate. Protein kinase activity was assayed as described in Materials and Methods with 50 μM [γ-^{32}P]ATP (1,350 dpm/pmol), 12 μg protein/ml BCE cytosolic preparation, and, where indicated, 200 μg/ml PS, 2 mM EGTA, 20 μg/ml DiC_8, and 50 nM PDBu or β-phorbol. Under these conditions, the rate of protein phosphorylation was constant for at least 60 min (data not shown). Protein phosphorylation is expressed as the amount of ^{32}P incorporated into histone during 50 min/50-μl aliquot. Each value given is the mean ± SEM for quadruplicate aliquots. All values have been corrected for basal protein kinase activity by subtracting the amount of ^{32}P incorporated in the absence of PS, EGTA, DiC_8, or phorbol compounds (4,200 ± 200 dpm).

ticular, DiC_4 is two to three orders of magnitude less potent than DiC_8 in activation of protein kinase C and in displacing PDBu from its receptor (Ebeling et al., 1985).

We therefore tested DiC_8 and DiC_4 for displacement of PDBu from its receptor in BCE cells. We found (Fig. 6) that DiC_8 displaced over 90% of the specific binding of PDBu, with 50% displacement occurring at 9 μM DiC_8. DiC_4, in accordance with its known low affinity for protein kinase C, did not displace PDBu from BCE cells at concentrations as high as 80 μM. Attempts to evaluate the effect of DiC_8 on BCE cell growth were unsuccessful. DiC_8, at concentrations of 100 μM or less, was rapidly metabolized in the cell cultures (as assessed by the ability of the culture medium to displace [^{3}H]PDBu binding), whereas higher concentrations of DiC_8 caused cell rounding and irreversible detachment.

These results are consistent with an interaction of PDBu with protein kinase C in BCE cells. To show that this enzyme exists in the BCE cell and that it is activated by PDBu, we prepared a cytosolic extract from BCE cells as described in Materials and Methods. In preliminary experiments (data not shown), we found that this preparation contained protein kinase activity that required both calcium and PS, i.e., protein kinase C activity. We tested the BCE cytosolic protein kinase C activity to determine whether it was activated by PDBu or diacylglycerol. Other studies have shown that both of these compounds increase the affinity of protein kinase C for calcium, causing it to become more active at suboptimal calcium concentrations (Nishizuka, 1984; Kishimoto et al., 1980). We assayed a BCE cytosolic preparation under conditions where submaximal protein kinase C activity was observed in the presence of PS and endogenous calcium (Fig. 7). This protein kinase C activity was increased by ~200% when DiC_8 was added. The DiC_8-stimulated protein kinase activity required PS and was abolished by EGTA. PDBu stimulated BCE cytosolic protein kinase C in a manner similar to DiC_8, while β-phorbol at the same concentration had no effect. Hydrolysis of radioactive ATP in these reactions, as determined by thin-layer chromatography (Doctrow and Lowenstein, 1985), was insignificant (<5%).

As discussed above, many mitogens induce PIP_2 hydrolysis in their target cells. It is therefore possible that HDGF might act via such a mechanism in BCE cells. If this were true, then the phorbol ester–induced inhibition observed by us might be a feedback inhibition of phospholipase C, similar to that proposed in other systems (Brock et al., 1985; Rosoff and Cantley, 1985). To test whether HDGF stimulates PIP_2 hydrolysis in BCE cells, we incubated [^{3}H]inositol prelabeled cells with and without HDGF and measured radioactive inositol phosphates as described in Materials and Methods. In the presence or absence of HDGF, we observed no changes in inositol phosphate levels compared with those present before test incubations. For example, in one experiment, the amount of inositol phosphate recovered from the cells was 236,600 ± 8,400 cpm/well inositol monophosphate and 12,400 ± 600 cpm/well inositol polyphosphates before and after 30 min incubation with HDGF (20 U) or PBS control solutions. Similarly, no changes in inositol phosphates were detected after incubation times of 15 s, 5 min, or 10 min. We also conducted experiments using cells prelabeled with radioactive arachidonic acid to detect diacylglycerol production as previously described (Kaibuchi et al., 1986). We found no change in diacylglycerol levels in the controls or in the HDGF-treated BCE cells.

Discussion

These results demonstrate that PDBu strongly attenuates the mitogenic response of BCE cells to the basic endothelial mitogen HDGF (Figs. 1, 3, and 4). We have also observed a similar effect on the response of these cells to retina-derived growth factor (Doctrow, S. R., and J. Sudhalter, unpublished observations), an acidic endothelial mitogen which, like HDGF and related factors, is angiogenic in vivo (D'Amore, 1982). These were unexpected findings, since PDBu and other protein kinase C activators are mitogenic or co-mitogenic for many other cell types (Dicker and Rozengurt, 1980; Kaibuchi et al., 1985; Gelfand et al., 1985; Bachrach et al., 1985; Balk et al., 1984) including the bovine aortic smooth muscle cells (Fig. 2 C) and BALB/c 3T3 fibroblasts (Table I) that we tested. (This is not, however, the first example of a cell whose growth is inhibited by phorbol diesters. These compounds inhibit the proliferation of HL-60 promyeloid leukemia cells, concomitantly causing them to differentiate into cells with the properties of normal macrophages [Huberman and Callahan, 1979; Ebeling et al., 1985].)

In contrast to its potent inhibition of BCE cell growth, PDBu had no effect on the proliferation of bovine aortic endothelial cells (Fig. 2 B). This lack of effect is not likely to be due to a protein kinase C deficiency in the aortic cells, since phorbol diesters at nanomolar concentrations induce a morphological change in bovine aortic endothelial cells and cause them to produce plasminogen activator (Gross et al., 1982). In addition, Mackie et al. (1986) have measured protein kinase C in extracts from bovine aortic endothelial and bovine pulminary arterial endothelial cells and report a specific activity (0.27 ± 0.02 nmol/mg protein per min) in agreement with that measured by us in BCE preparations (~0.2 nmol/mg protein per min). While the reason for the

difference that we observe between BCE cells and bovine aortic endothelial cells is not clear, it may indicate some specificity of PDBu-mediated growth inhibition for small vessel endothelium.

A number of our observations suggest that PDBu interacts with BCE cells through protein kinase C. Inhibition of BCE cell growth occurred at PDBu concentrations well within the range of those shown to increase protein kinase C in other systems (Rozengurt et al., 1983; Kikkawa et al., 1983). Various phorbol compounds inhibited HDGF-mediated growth with an order of potency that correlated very closely with their known relative potencies as activators of isolated protein kinase C (Table II).

Our binding studies (Fig. 5) revealed that PDBu interacts with a specific, saturable receptor on BCE cells. The apparent dissociation constant for this interaction (8 nM) agrees with the K_{50} for the inhibition of BCE cell growth (6 $\pm$ 4 nM). It is therefore likely that the receptor detected in our binding studies mediates the growth effect. The PDBu receptor in BCE cells has affinity for the protein kinase C–activating diacylglycerol DiC_8, but not for the inactive DiC_4 (Fig. 6). The concentration range of DiC_8 required to compete with PDBu for binding to BCE cells is equivalent to that needed to displace PDBu from A431 cells (Davis et al., 1985) and HL-60 cells (Ebeling et al., 1985), two cell types in which PDBu is thought to exert its effects via protein kinase C. In addition, similar concentrations of DiC_8 have been shown to activate protein kinase C in intact platelets (Lapetina et al., 1985). Because an sn-1,2-diacylglycerol is the postulated endogenous activator of protein kinase C (Nishizuka, 1984), the affinity of the PDBu receptor in BCE cells for DiC_8 is perhaps the most important piece of evidence that this receptor is indeed protein kinase C. This interpretation is strengthened by the observation that DiC_4, a very weak protein kinase C activator (Ebeling et al., 1985; Lapetina et al., 1985), did not displace PDBu binding even though both DiC_4 and PDBu are dibutyryl esters.

This study also demonstrates that protein kinase C can be detected enzymatically in a BCE cytosolic preparation and that the enzyme is activated by PDBu (Fig. 7). This was not unexpected since protein kinase C has been found in many different tissues. However, our data eliminate the remote possibility that BCE cells are either lacking in protein kinase C or contain a similar enzyme that is unresponsive to diacylglycerols or phorbol diesters.

We were unable to detect HDGF-induced changes in inositol phosphate or diacylglycerol levels in BCE cells. We conclude that HDGF, while stimulating BCE cell growth, does not seem to cause PIP_2 hydrolysis under our conditions. Although we used techniques that have successfully demonstrated mitogen-induced PIP_2 hydrolysis in other systems, we cannot exclude the possibility that a functionally important, but minor, pool of messenger is being masked in our experiments. But, presently, we have no evidence that HDGF activates protein kinase C in these cells, which is consistent with our observation that HDGF and phorbol diesters do not have a synergistic or additive effect in our experiments.

The molecular mechanisms of BCE cell growth stimulation by HDGF and its inhibition by protein kinase C remain unknown. Our data indicate a protein kinase C–mediated uncoupling of HDGF from its intracellular effects, essentially desensitizing the endothelial cells to mitogens. This desensitization might involve down-regulation of HDGF receptors or disruption of one or more of the intracellular events required to transduce the mitogenic signal to the nucleus. It is not likely that PDBu competes directly with a single step in the normal signal transduction cascade of HDGF, because very high amounts of growth factor do not overcome inhibition by even subsaturating concentrations of PDBu (Fig. 3). PDBu-induced down-regulation of protein kinase C, a phenomenon reported in certain other cells (Kaibuchi et al., 1986), does not explain our results, since PDBu pretreatment completely inhibits DNA synthesis in BCE cells without affecting their [^{3}H]PDBu binding capacity (data not shown).

A process that can render capillary endothelial cells insensitive to mitogens is likely to be crucial to the formation of functional capillaries. Angiogenesis in vivo requires not only endothelial cell proliferation, but also organization of these cells into nonproliferating tubes (Folkman et al., 1982). When capillary endothelial cells are induced to form tubes in culture, they have a greatly reduced rate of mitosis compared with cells that are not organizing in this fashion (Folkman and Haudenschild, 1980; Madri, et al., 1983). In vivo, growing capillary networks consist of proliferating endothelial cells that are often in close proximity to nonproliferating, tube-forming cells (Ausprunk and Folkman, 1977). Thus, mechanisms must exist for locally suppressing the growth of certain endothelial cells even in the presence of significant amounts of mitogen. It is possible that protein kinase C activation is an intracellular signal inducing endothelial cells to become desensitized to angiogenic endothelial mitogens and perhaps, as discussed below, to organize into tubes. Inasmuch as tube formation is considered a more differentiated state than proliferation for endothelial cells (Madri et al., 1983), protein kinase C activation in response to specific localized signals (e.g., extracellular matrix composition) may promote differentiation in these cells.

Previously reported effects of TPA on capillary endothelial cells support a phorbol diester–induced differentiation. When a capillary endothelial cell monolayer on the surface of a collagen gel is treated with TPA, the cells organize into capillary-like tubes inside the gel (Montesano and Orci, 1985). TPA causes capillary endothelial cell monolayers to produce proteases, which may allow them to modify their substratum and invade tissues during certain phases of angiogenesis (Gross et al., 1982). The TPA-induced effects leading to tube formation and protease production occur independently of cell growth, and therefore are consistent with our present observation that protein kinase C activation suppresses the proliferation of these cells in response to mitogens. Most recently, Lombardi et al. (1986) have demonstrated that TPA induces cultured BCE cells to exhibit diaphragmed fenestrae, a differentiated characteristic that was previously difficult to demonstrate in vitro.

These previous reports, together with our present findings, suggest that protein kinase C activation may be one intracellular signal for switching capillary endothelial cells from a proliferative into a more differentiated state. The precise role played by protein kinase C in angiogenesis is not easily predicted by in vitro experiments. It is highly unlikely that indiscriminate activation of this kinase in a population of endothelial cells would be sufficient to trigger the construction of a complete vascular system in vivo, especially since it would inhibit their proliferation. However, selective modula-

tion of this enzyme during appropriate stages of angiogenesis might help to direct this complex process. We speculate that protein kinase C–induced uncoupling of mitogen action is one important step promoting endothelial cell organization into mature, nongrowing capillaries.

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References

Ausprunk, D., and J. Folkman. 1977. Migration and proliferation of endothelial cells in preformed and newly formed blood vessels during tumor angiogenesis. *Microvasc. Res.* 14:53–65.

Bachrach, L. K., M. C. Eggo, W. W. Mak, and G. W. Burrow. 1985. Phorbol esters stimulate growth and inhibit differentiation in cultured thyroid cells. *Endocrinology.* 116:1603–1609.

Balk, S. D., A. Morisi, and H. S. Gunther. 1984. Phorbol 12,13-myristate acetate, ionomycin, or ouabain and raised extracellular magnesium induced proliferation of chicken heart mesenchymal cells. *Proc. Natl. Acad. Sci. USA.* 81:6418–6421.

Berridge, M. J. 1983. Rapid accumulation of inositol triphosphate reveals that agonists hydrolyze polyphosphoinositides instead of phosphatidylinositol. *Biochem. J.* 212:849–858.

Berridge, M. J. 1984. Inositol triphosphate and diacylglycerol as second messengers. *Biochem. J.* 220:345–360.

Berridge, M. J., J. P. Heslop, R. F. Irvine, and K. D. Brown. 1984. Inositol triphosphate formation and calcium mobilization in Swiss 3T3 cells in response to platelet-derived growth factor. *Biochem. J.* 222:195–201.

Berridge, M. J. and R. F. Irvine. 1984. Inositol triphosphate, a novel second messenger in cellular signal transduction. *Nature (Lond.).* 312:315–321.

Besterman, J. M., S. P. Watson, and P. Cuatrecasas. 1986. Lack of association of epidermal growth factor-, insulin-, and serum-induced mitogenesis with stimulation of phosphoinositide degradation in Balb c/3T3 fibroblasts. *J. Biol. Chem.* 261:723–727.

Brock, T. A., S. E. Rittenhouse, C. W. Powers, L. Ekstein, M. Gimbrone, and R. W. Alexander. 1985. Phorbol ester and 1-oleoyl-2-acetylglycerol inhibit angiogensin activation of phospholipase C in cultured VSM cells. *J. Biol. Chem.* 260:14158–14162.

Castagna, M., Y. Takai, K. Kaibuchi, K. Sano, A. Kikkawa, and Y. Nishizuka. 1982. Direct activation of calcium activated phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J. Biol. Chem.* 257:7847–7851.

D'Amore, P. A. 1982. Purification of a retina-derived endothelial cell mitogen/angiogenic factor. *J. Cell Biol.* 95(2, Pt. 2):192a. (Abstr.)

D'Amore, P. A., and M. Klagsbrun. 1984. Endothelial cell mitogens derived from retina and hypothalamus: biochemical and biological similarities. *J. Cell Biol.* 99:1545–1549.

D'Amore, P. A., B. M. Glaser, S. K. Brunson, and A. J. Fensleau. 1981. Angiogenic activity from bovine retina: partial purification and characterization. *Proc. Natl. Acad. Sci. USA.* 78:3068–3072.

Davis, R. J., B. R. Ganong, R. M. Bell, and M. P. Czech. 1985. sn-1,2-Dioctanoylglycerol. A cell-permeable diacylglycerol that mimics phorbol diester action on the epidermal growth factor receptor and mitogenesis. *J. Biol. Chem.* 260:1562–1566.

Dicker, P., and E. Rozengurt. 1980. Phorbol esters and vasopressin stimulate DNA synthesis by a common mechanism. *Nature (Lond.).* 287:607–612.

Doctrow, S. R., and J. M. Lowenstein. 1985. Adenosine and 5'chloro 5'-deoxyadenosine inhibit the phosphorylation of phosphatidylinositol and myosin light chain in calf aorta smooth muscle. *J. Biol. Chem.* 260:3469–3476.

Ebeling, J. G., G. R. Vanderbark, L. J. Kuhn, B. R. Ganong, R. M. Bell, and J. E. Neidel. 1985. Diacylglycerols mimic phorbol diester induction of leukemic cell differentiation. *Proc. Natl. Acad. Sci. USA.* 82:815–819.

Folkman, J. 1982. Angiogenesis: initiation and control. *Ann. N.Y. Acad. Sci.* 401:212–227.

Folkman, J., and C. C. Haudenschild. 1980. Angiogenesis *in vitro*. *Nature (Lond.).* 288:551–556.

Folkman, J., C. C. Haudenschild, and B. R. Zetter. 1979. Long-term culture of capillary endothelial cells. *Proc. Natl. Acad. Sci. USA.* 76:5217–5221.

Folkman, J., C. C. Haudenschild, and B. Zetter. 1982. Capillary endothelial cells: growth control and differentiation. *Cold Spring Harbor Conf. Cell Proliferation.* 9:509–521.

Ganong, B. R., and R. M. Bell. 1985. Diacylglycerols function as bioregulators of protein kinase C and cellular responses. *Fed. Proc.* 44:650.

Gelfand, E. W., R. K. Cheung, G. B. Mills, and S. Grinstein. 1985. Mitogens trigger a calcium-dependent signal for proliferation in phorbol ester-treated lymphocytes. *Nature (Lond.).* 315:419–420.

Gitlin, J. D., and P. A. D'Amore. 1983. Culture of retinal capillary cells using selective growth media. *Microvasc. Res.* 26:74–80.

Gospodarowicz, D., J. Cheng, G. M. Lui, A. Baird, and P. Bohlen. 1984. Isolation of brain fibroblast growth factor by heparin-Sepharose affinity chromatography: identity with pituitary fibroblast growth factor. *Proc. Natl. Acad. Sci. USA.* 81:6963–6967.

Gross, J. L., D. Moscatelli, E. A. Jaffe, and D. B. Rifkin. 1982. Plasminogen activator and collagenase production by cultured capillary endothelial cells. *J. Cell Biol.* 95:974–981.

Huberman, E., and M. Callaham. 1979. Induction of terminal differentiation in human pro-myelocytic leukemia cells by tumor-promoting agents. *Proc. Natl. Acad. Sci. USA.* 76:1293–1297.

Kaibuchi, K., Y. Takai, and Y. Nishizuka. 1985. Protein kinase C and calcium ion in mitogenic response of macrophage-depleted human peripheral lymphocytes. *J. Biol. Chem.* 260:1366–1369.

Kaibuchi, K., T. Tsuda, A. Kikuchi, T. Tanimoto, T. Yamashita, and Y. Takai. 1986. Possible involvement of protein kinase C and calcium ion in growth factor-induced expression of c-myc oncogene in Swiss 3T3 fibroblasts. *J. Biol. Chem.* 261:1187–1192.

Kates, M. 1972. Techniques of Lipidology. Elsevier Science Publishing Co., Inc. New York. 358.

Kikkawa, U., Y. Takai, Y. Tanaka, R. Mikaye, and Y. Nishizuka. 1983. Protein kinase C as a possible receptor protein of tumor-promoting phorbol esters. *J. Biol. Chem.* 258:11442–11445.

Kishimoto, A., Y. Takai, T. Mori, U. Kikkawa, and Y. Nishizuka. 1980. Activation of calcium and phospholipid-dependent protein kinase by diacylglycerol, its possible relation to phosphatidylinositol turnover. *J. Biol. Chem.* 255:2273–2276.

Klagsbrun, M., J. Sasse, R. Sullivan, and J. A. Smith. 1986. Human tumor cells synthesize an endothelial cell growth factor that is structurally related to basic fibroblast growth factor. *Proc. Natl. Acad. Sci. USA.* 83:2448–2452.

Klagsbrun, M., and Y. Shing. 1985. Heparin affinity of anionic and cationic capillary endothelial cell growth factors: analysis of hypothalamus-derived growth factors and fibroblast growth factors. *Proc. Natl. Acad. Sci. USA.* 82: 805–809.

Lapetina, E. G., B. Reep, B. R. Ganong, and R. M. Bell. 1985. Exogenous sn-1,2-diacylglycerols containing saturated fatty acids function as bioregulators of protein kinase C in human platelets. *J. Biol. Chem.* 260:1358–1361.

Lobb, R., J. Sasse, R. Sullivan, Y. Shing, P. D'Amore, J. Jacobs, and M. Klagsbrun. 1986. Purification and characterization of heparin-binding endothelial cell growth factors. *J. Biol. Chem.* 261:1924–1928.

Lombardi, T., R. Montesano, M. B. Furie, S. C. Silverstein, and L. Orci. 1986. Endothelial diaphragmed fenestrae: in vitro modulation by phorbol myristate acetate. *J. Cell Biol.* 102:1965–1970.

Maciag, T., T. Mehlan, R. Friesel, and A. B. Schreiber. 1984. Heparin binds endothelial cell growth factor, the principal endothelial cell mitogen in bovine brain. *Science (Wash. DC).* 225:932–935.

Mackie, K., Y. Lai, A. C. Nairn, P. Greengard, B. R. Pitt, and J. S. Lazo. 1986. Protein phosphorylation in cultured endothelial cells. *J. Cell. Physiol.* 128:367–374.

Madri, J. A., S. K. Williams, T. Wyatt, and C. Mezzio. 1983. Capillary endothelial cell cultures: phenotypic modulation by matrix components. *J. Cell Biol.* 97:153–165.

Michell, R. H. 1982. Inositol lipid metabolism in dividing and differentiating cells. *Cell Calcium.* 3:429–440.

Montesano, R., and L. Orci. 1985. Tumor-promoting phorbol esters induce angiogenesis *in vitro*. *Cell.* 42:469–477.

Nishizuka, Y. 1984. The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature (Lond.).* 308:693–698.

Noguchi, M., H. Adachi, J. D. Gardner, and R. T. Jensen. 1985. Calcium-activated, phospholipid-dependent protein kinase in pancreatic acinar cells. *Am. J. Physiol.* 248:G692-G701.

Rosoff, P. M., and L. Cantley. 1985. Lipopolysaccharide and phorbol esters induce differentiation but have opposite effects on phosphatidylinositol turnover and calcium mobilization in 70Z/3 lymphocytes. *J. Biol. Chem.* 260:9209–9215.

Rozengurt, E., A. Rodriguez-Pena, M. Coombs, and J. Sinnet-Smith. 1984. Diacylglycerol stimulates DNA synthesis and cell division in mouse 3T3 cells: role of calcium-sensitive phospholipid-dependent protein kinase. *Proc. Natl. Acad. Sci. USA.* 81:5748–5752.

Rozengurt, E., M. Rodriguez-Pena, and K. A. Smith. 1983. Phorbol esters, phospholipase C, and growth factors rapidly stimulate the phosphorylation of a M_r 80,000 protein in intact quiescent 3T3 cells. *Proc. Natl. Acad. Sci. USA.* 80:7244–7248.

Shing, Y., J. Folkman, C. Haudenschild, D. Lund, R. Crum, and M. Klagsbrun. 1985. Angiogenesis is stimulated by a tumor-derived endothelial cell growth factor. *J. Cell. Biochem.* 29:275–285.

Shing, Y., J. Folkman, R. Sullivan, C. Butterfield, J. Murray, and M. Klagsbrun. 1984. Heparin affinity: purification of a tumor-derived capillary endothelial cell growth factor. *Science (Wash. DC).* 223:1296–1298.

Sullivan, R., and M. Klagsbrun. 1985. Purification of cartilage-derived growth factor by heparin affinity chromatography. *J. Biol. Chem.* 260:2399–2403.

Thomas, K. A., and G. Giminez-Gallego. 1986. Fibroblast growth factor: broad spectrum mitogens with potent angiogenic activity. *Trends Biochem. Sci.* 11:81–84.

Voyta, J. C., D. P. Via, C. E. Butterfield, and B. R. Zetter. 1984. Identification and isolation of endothelial cells based on their increased uptake of acetylated-low density lipoprotein. *J. Cell Biol.* 99:2034–2040.