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 (Kd = 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 Kd 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) 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 diacetyl glycerol 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.

**Materials and Methods**

**Materials**

Tris base, Hepes, EGTA, EDTA, p-toluene sulfonyl fluoride, aprotonin, BSA, and histone (type III) were purchased from Sigma Chemical Co. (St. Louis, MO). Phorbol compounds were from Sigma Chemical Co. or from LC Biochemicals (Piscataway, NJ). [γ-32P]ATP, [20-3H(N)]phorbol 12,13-dibutyrate, [3H]-H-thymidine, [2-3H(N)]myo-inositol, [5,6,9,11,12,14,15-3H]arachidonic acid, and [3H]thymidine 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 (DiC4) and phosphate (PS) were obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). sn-1,2-Dibutyrylglycerol (DiC4) 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 diacetyl glycerol or phorbol diester in stock solutions were measured by an ester determination procedure using alkaline hydroxyamine 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., 1994). Unless otherwise specified, the medium was DME with 10% calf serum. For experiments measuring the incorporation of [3H]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, Elkhart, IN). Unless otherwise indicated, the medium was DME with 1% calf serum and 0.1% MeSO. Cells were incubated with [3H]-H-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 with 1 ml of cold acetic acid/methanol (1:3) and twice with 10 ml of cold 10% TCA. The precipitates were then washed with water and dissolved in three successive 0.5-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.

**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.
PDBu Binding to BCE Cells

Cells were plated in gelatinized 24-well plates (16-mm diameter wells) at 15,000 cells/well and incubated for 3–4 d in DMEM-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 \(\mu\)M unlabeled PDBu. Nonradioactive PDBu, when present, was added as a solution in Me\(_2\)SO 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\(_2\)SO 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 were 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, then 1 ml extraction buffer was added, and the samples were treated with a proteinase K 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 \(\mu\)l chloroform and 800 \(\mu\)l 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 autoradiography.

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 \(\mu\)M. 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 of even suboptimal (15 nM) PDBu was
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-tetradecahydro-
Table I. Stimulation of BALB/c 3T3 Cell DNA Synthesis by PDBu or HDGF

| Treatment          | [3H]Thymidine incorporation (dpm) |
|--------------------|----------------------------------|
| None               | 949 ± 74                         |
| PDBu               | 59,288 ± 1,733                   |
| HDGF               | 33,198 ± 1,084                   |
| PDBu + HDGF        | 111,186 ± 6,350                  |

Incorporation of [3H]thymidine by quiescent BALB/c 3T3 cells was determined as described previously (Klagsbrun and Shing, 1985). The wells contained 0.1% Me$_2$SO and, where indicated, 30 nM PDBu and 4 U per ml HDGF. Each value represents the mean ± SEM for quadruplicate wells.

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) | Percent protein kinase C activation (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 α-Phorbol 12, 13-didecanoate | 76,900 ± 3,500  | 1                   | 0                                                         | 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 15,000. 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).
protein kinase C in BCE cells. To show that this enzyme
protein kinase activity by subtracting the amount of ^32P incorpo-
ating 50 min/50-ktl aliquot. Each value given is the mean + SEM

Figure 7. Activation of BCE
cytosolic protein kinase C by
DiC₄ or phorbol dibutyrate.
Protein kinase activity was as-
sayed as described in Mate-
rials and Methods with 50 μM
[y-^32P]ATP (1,350 dpm/pmol),
12 μg protein/ml BCE cyto-
solic preparation, and, where
indicated, 200 μg/ml PS, 2 mM
EGTA, 20 μg/ml DiC₄, and
50 nM PDBu or β-phorbol.
Under these conditions, the
rate of protein phosphoryla-
tion was constant for at least
60 min (data not shown). Pro-
tein phosphorylation is ex-
pressed as the amount of ^32P incorpo-
rated 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 ^32P incorpo-
rated in the absence of PS, EGTA, DiC₄, or phorbol compounds
(4,200 ± 200 dpm).

ticular, DiC₄ is two to three orders of magnitude less potent
than DiC₈ in activation of protein kinase C and in displac-
ing PDBu from its receptor (Ebeling et al., 1985).

We therefore tested DiC₄ and DiC₈ for displacement of
PDBu from its receptor in BCE cells. We found (Fig. 6) that
DiC₄ displaced over 90% of the specific binding of PDBu,
with 50% displacement occurring at 9 μM DiC₄. DiC₄, 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 DiC₄ on
BCE cell growth were unsuccessful. DiC₄, at concentra-
tions of 100 μM or less, was rapidly metabolized in the cell
cultures (as assessed by the ability of the culture medium
to displace [^3H]PDBu binding), whereas higher concentra-
tions of DiC₄ caused cell rounding and irreversible de-
tachment.

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., pro-
tein 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 condi-
tions where submaximal protein kinase C activity was ob-
served in the presence of PS and endogenous calcium (Fig.
7). This protein kinase C activity was increased by ~200%
when DiC₄ was added. The DiC₄-stimulated protein kinase
activity required PS and was abolished by EGTA. PDBu
stimulated BCE cytosolic protein kinase C in a manner simi-
lar to DiC₄, while β-phorbol at the same concentration had
no effect. Hydrolysis of radioactive ATP in these reactions,
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 \( K_d \) for the inhibition of BCE cell growth (6 ± 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\(_8\), a very weak protein kinase C activator (Ebeling et al., 1985; Lapetina et al., 1985), did not displace PDBu binding even though both DiC\(_8\) 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 (Auspfrunk 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-
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