Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 5961–5965, August 1988
Cell Biology

Angiogenin activates endothelial cell phospholipase C

(diacylglycerol/second messenger/angiogenesis)

ROY BICKNELL AND BERT L. VALLEE

Center for Biochemical and Biophysical Sciences and Medicine, Harvard Medical School, Boston, MA 02115

Contributed by Bert L. Vallee, May 2, 1988

ABSTRACT **Low concentrations of angiogenin activate the inositol-specific phospholipase C of cultured pulmonary artery, umbilical vein, and capillary endothelial cells, promoting a transient increase in the intracellular levels of 1,2-diacylglycerol and inositol trisphosphate. The response is strongly dose dependent with a maximum in the ng/ml concentration range and, for some cell lines, a marked decrease at concentrations >1 ng/ml; e.g., arterial endothelial cells respond weakly to angiogenin concentrations comparable to that in normal human plasma (≈400 ng/ml). Chemical modification of the active site of angiogenin or inhibition with placental ribonuclease inhibitor abolishes its activation of endothelial cell phospholipase C; this correlates with the concomitant loss of both intrinsic ribonucleolytic and angiogenic activity. The response to low concentrations of angiogenin is consistent with its potency of inducing vascularization in classical angiogenesis assays. *In vivo*, endothelial cells are exposed to concentrations of angiogenin higher than that required to elicit a cellular response; it seems likely, therefore, that expression of a surface receptor or some other process must be rate limiting in the cellular response.**

Angiogenin, secreted by HT-29 human colon adenocarcinoma cells (1), is a potent inducer of vascular development in the chicken chorioallantoic membrane (1, 2). The primary structure of this 14-kDa protein exhibits significant sequence homology to pancreatic ribonuclease (3). Moreover, it has intrinsic ribonucleolytic activity distinct from that of "classic" ribonucleases (4). Angiogenin is a very effective inhibitor of cell-free protein synthesis in the rabbit reticulocyte lysate. Inhibition is a direct result of specific angiogenin cleavage of ribosomal RNA and consequent inactivation of the protein synthesis machinery (5). To date there is no evidence that angiogenin either inhibits protein synthesis *in vivo* or enters undamaged cells; hence, the physiological significance of these findings remains to be clarified.

Several other structurally characterized proteins—notably, tumor necrosis factor, transforming growth factors α and β, epidermal growth factor, as well as acidic and basic fibroblast growth factor—initially identified on the basis of other biological activities, have also been shown to be angiogenic (6–8). All of these proteins have been the subject of extensive cell biological research and the details of their interactions with various cell types have been carefully documented. In contrast, little is known about the interaction of angiogenin with cells, including the specificity of this interaction, the nature of the cellular response, how this relates to the ribonucleolytic activity of angiogenin, and how this ultimately leads to vascularization. In particular, it was not known whether angiogenin interacts directly or indirectly with the capillary endothelium. The results presented here demonstrate that angiogenin activates endothelial cell phospholipase C at concentrations well below that in normal human plasma but comparable to those that elicit angiogenic responses in the chorioallantoic membrane assay.

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MATERIALS AND METHODS

Materials. Angiogenin was isolated from either normal human plasma (9) or medium conditioned by baby hamster kidney cells genetically altered to express angiogenin (10). The responses to plasma-derived and expressed angiogenin were indistinguishable in all experiments. Stock solutions of angiogenin were free of endotoxins (<1 ng/ml in mg/ml stocks) by the *Limulus* amoebocyte assay (11) using the Sigma E-TOXATE kit. Low concentration solutions of angiogenin were prepared from mg/ml stocks by dilution into Hanks' balanced salt solution (without bicarbonate and phenol red) immediately before use. Caution in this regard is essential since angiogenin is rapidly lost from dilute (<μg/ml) solutions by adsorption to the walls of containers (W. F. Heath and M. D. Bond, personal communication). Chemically modified derivatives of angiogenin were prepared as described (12).

[*inositol*-2-^{3}H]Phosphatidylinositol 4,5-bisphosphate (PtdInsP_2) (4 Ci/mmol; 1 Ci = 37 GBq), L-α-[*myo-inositol*-2-^{3}H] phosphatidylinositol (PtdIns) (10 Ci/mmol), and D-[*inositol*-2-^{3}H]inositol 1,4,5-trisphosphate (InsP_3) (3.3 Ci/mmol) were from New England Nuclear, [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid was from either New England Nuclear (190–240 Ci/mmol) or Research Products International, Mt. Prospect, IL (130–180 Ci/mmol). *myo*-[2-^{3}H]Inositol was either from New England Nuclear (12.8 Ci/mmol) or Amersham (16.6 Ci/mmol). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, glutamine, fungizone, and gentamicin sulfate were from M. A. Bioproducts, Walkersville, MD; bradykinin was from Vega Chemical, Tucson, AZ; and L-α-1,2-dioleoylglycerol, PtdIns, and lysophosphatidylinositol (lyso-PtdIns) from Avanti Polar Lipids, Birmingham, AL. Endothelial cell growth supplement from bovine neural tissue was from Sigma.

Cell Culture. Human umbilical vein endothelial (HUVE) CRL 1730 and calf pulmonary artery endothelial (CPAE) CCL 209 cells were obtained from the American Type Culture Collection. Bovine adrenal capillary endothelial (BACE) cells isolated by published procedures (13) were a generous gift of William Heath. CPAE cells were cultured in DMEM supplemented with 20% fetal bovine serum/2 mM glutamine/fungizone (1 μg/ml)/gentamicin sulfate (5 μg/ml). Cells between passages 16 and 23 inclusive were seeded into 35-mm wells and used at confluence (4–6 days). HUVE and BACE cells were cultured on gelatin-coated Petri dishes in DMEM supplemented with 10% fetal bovine serum/4 mM glutamine/heparin (90 μg/ml)/gentamicin sulfate (5 μg/ml)/

Abbreviations: CPAE, calf pulmonary artery endothelial; BACE, bovine adrenal capillary endothelial; HUVE, human umbilical vein endothelial; PtdIns, phosphatidylinositol; PtdIns$P$, phosphatidylinositol 4-phosphate; PtdInsP_2, phosphatidylinositol 4,5-bisphosphate; lyso-PtdIns, lysophosphatidylinositol; InsP_3, inositol trisphosphate; InsP_2, inositol bisphosphate; Ins$P$, inositol monophosphate.

fungizone (0.5 μg/ml)/endothelial cell growth supplement (30 μg/ml). All cells were passaged at confluence by harvesting with trypsin/EDTA and seeding at a 1:3 ratio.

Phospholipid Labeling, Extraction, and Isolation. Cell cultures were labeled at confluence by incubation with either [^{3}H]arachidonic acid (5 μCi/ml) in 10% fetal bovine serum supplemented basic medium for 48 hr or with *myo*-[^{3}H]-inositol (40 μCi/ml) in standard growth medium for 72 hr. In the presence of growth supplement, BACE cells were markedly elongated; however, after 48 hr in the absence of growth supplement they formed a characteristic endothelial cell "cobblestone" monolayer. All experiments were performed with monolayers of the cobblestone morphology. Unincorporated label was removed by washing cultures two times with unsupplemented DMEM, followed by incubation in this medium in 5% CO_2/95% air for 3 hr prior to exposure to agonist. For InsP_3 determinations, the cells were incubated for a further 30 min in Hanks' balanced salt solution containing 50 mM lithium chloride. The cells were further washed with Hanks' balanced salt solution immediately before exposure to agonist in the same medium. The reaction was terminated by aspiration of the agonist-containing medium, followed by immediate addition of 1 ml of organic extraction medium. Neutral lipids were extracted with chloroform/methanol (1:1) and phospholipids were extracted with chloroform/methanol/conc. HCl (1:2:0.05). Extracts were treated as described (14).

Phospholipids were resolved by thin-layer chromatography on Whatman LK5D linear-K silica gel plates dipped in 1% potassium oxalate/2 mM EDTA with a chloroform/methanol/4 M NH_4OH (9:7:2) solvent (15). Neutral lipids were resolved on silica gel G plates (14) and were visualized by iodine staining, with L-α-1,2-dioleoylglycerol as standard. Phospholipids were visualized by autoradiography on Kodak X-OMAT AR film after treatment with EN^{3}HANCE (New England Nuclear). Tritiated PtdIns and PtdInsP_2 (New England Nuclear) and unlabeled lyso-PtdIns (Avanti Polar Lipids) (visualized by iodine stain) were used as standards. Lipids were quantitated by liquid scintillation counting. Aqueous extracts containing labeled inositol phosphates were resolved by ion-exchange chromatography (16).

RESULTS

Time Course and Dose Response of Angiogenin-Stimulated Diacylglycerol Formation in Cultured CPAE Cells. Stimulation of CPAE cell cultures by angiogenin (1 ng/ml) at 37°C induces an increase in diacylglycerol that is detectable after 30 s, peaks in 2.5 min at 59% ± 13% above controls, and returns to the resting level within 20 min (Fig. 1*A*). The time course for diacylglycerol formation on stimulation with much higher concentrations of angiogenin (500 ng/ml) is similar although the extent of the response is 10 times less (Fig. 1*B*). Fig. 2*A* illustrates the dose dependence of the diacylglycerol increase. With this cell line, the threshold for angiogenin-induced diacylglycerol formation is 0.01 ng/ml, and the response peaks at 1 ng/ml and decreases sharply at higher concentrations of angiogenin, such that only a small increase in diacylglycerol levels is seen with angiogenin concentrations of ≥100 ng/ml. In these experiments, bradykinin (1 μg/ml) served as a positive control agonist (17). The magnitude of the induced increases in diacylglycerol for angiogenin (1 ng/ml) (1140 ± 60 cpm above controls) and bradykinin (1 μg/ml) (830 ± 70 cpm above controls) is similar.

Angiogenin-Stimulated Diacylglycerol Formation in Other Endothelial Cell Lines. Angiogenin also induces diacylglycerol formation in other endothelial cell lines. Fig. 2*B* shows a dose–response curve with a capillary (BACE) cell line. Again, low concentrations of angiogenin elicit a cellular response, which in this case peaks with angiogenin at 0.1

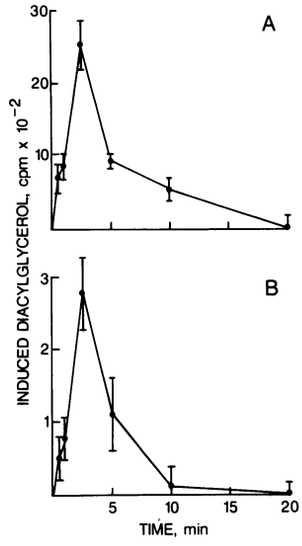

FIG. 1. Time course of angiogenin-stimulated diacylglycerol increase in CPAE cells at 37°C. The angiogenin concentration was either 1 ng/ml (*A*) or 500 ng/ml (*B*). Each point represents the mean ± SEM (n = 4). The amount of diacylglycerol radioactivity in controls was 4250 ± 220 cpm (*A*) and 2750 ± 30 cpm (*B*).

ng/ml. Although the response decreases somewhat at higher concentrations of angiogenin, it remains substantial, with a concentration of 100 ng/ml in this instance. In contrast, higher concentrations of angiogenin (100–1000 ng/ml) are required to induce a significant increase (75% ± 4% above controls) in the diacylglycerol level of the HUVE cell line (Fig. 2*C*), and there is no evidence of a decrease in the response at concentrations of angiogenin up to 1 μg/ml.

Effect of Chemical Modification of Angiogenin on the Activation of Phospholipase C. Angiogenin was chemically modified to abolish its ribonucleolytic activity by bromoacetate alkylation of one or both of the active site histidine residues (His-13 and His-114) (4, 12) to determine whether this enzymatic activity is needed to elicit a diacylglycerol response. Table 1 shows that carboxymethylation of His-13 and/or His-114 abolishes the capacity of angiogenin to induce an increase in CPAE cellular diacylglycerol. Angiogenin not

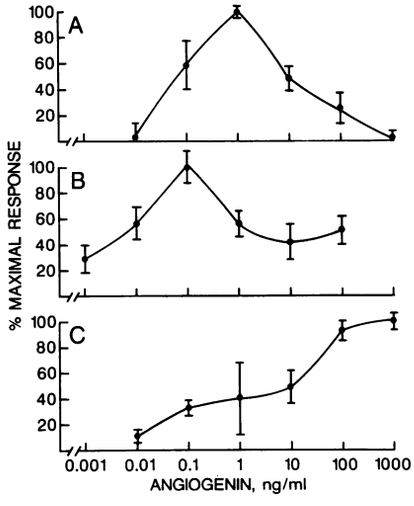

FIG. 2. Dose response of angiogenin-stimulated diacylglycerol increase in cultured endothelial cells at 37°C. (*A*) CPAE cells, 2.5 min exposure; peak (1 ng of angiogenin per ml), 2440 ± 60; control, 1300 ± 110 cpm. (*B*) BACE cells, 1 min exposure; peak (0.1 ng of angiogenin per ml), 10700 ± 350; control, 7440 ± 1080 cpm. (*C*) HUVE cells, 2 min exposure; peak (1 μg of angiogenin per ml), 3970 ± 80; control, 2270 ± 290 cpm. Each point represents the mean ± SEM (n = 4).

Table 1. Effect of chemical modification and inhibition of angiogenin on its induction of diacylglycerol in cultured CPAE cells

Agonist	Induced diacylglycerol, cpm
Angiogenin (1 ng/ml)	2300 ± 450
Bromoacetate modified (both histidines) (1 ng/ml)*	100 ± 370
Bromoacetate modified (one histidine) (1 ng/ml)*	430 ± 415
Angiogenin (1 ng/ml) + PRI (10 ng/ml)†	10 ± 390

Conditions: Cells were exposed to agonist for 1 min. cpm are mean (above control of 2250 ± 270 cpm) ± SEM (n = 5).
*See ref. 12.
†Angiogenin and placental ribonuclease inhibitor (PRI) were mixed before addition to the cells.

exposed to the modification reagent but treated in an otherwise identical way elicits the usual response. Placental ribonuclease inhibitor (10 ng/ml) also completely abolishes the capacity of angiogenin (1 ng/ml) to elicit a response.

Ribonuclease A, although homologous with angiogenin (3), does not stimulate diacylglycerol formation at concentrations ranging from 1 to 100 ng/ml (data not shown).

Angiogenin-Stimulated Inositol Phospholipid Turnover. Inositol-containing phospholipids of BACE cell membranes were labeled with *myo*-[^{3}H]inositol and the cellular phospholipid content was determined before and after exposure to angiogenin (0.1 ng/ml) for various periods of time. BACE cells were chosen for detailed study because of their substantially greater incorporation of *myo*-inositol label compared to that of CPAE and HUVE cells (data not shown). Angiogenin promotes a decrease in cellular PtdInsP_2, which reaches a minimum at 1 min (an 18% decrease) and returns to the resting value within 2.5 min. Over the same time period, intracellular phosphatidylinositol 4-phosphate (PtdIns$P$) does not change significantly (Fig. 3A). The decrease in PtdInsP_2 is accompanied by a marked decrease in intracellular PtdIns: after 1 min of exposure to angiogenin, the PtdIns fraction had 30,000 cpm less than that of the controls (a 21% decrease). However, in contrast to PtdInsP_2, the PtdIns level does not rapidly return to that of unstimulated cells in the continued presence of angiogenin (Fig. 3B). A similar 20% decrease in PtdIns is seen over a period of at least 5 min after exposure of CPAE cells to angiogenin (1 ng/ml) (data not shown). Thus, it appears that angiogenin stimulates a burst of diacylglycerol (peak at 1 min, 1 ng of angiogenin per ml) by phospholipase C catalyzed hydrolysis of PtdInsP_2 concomitantly with a slower release of diacylglycerol by phospholipase C hydrolysis of PtdIns.

Fig. 3C shows that angiogenin (0.1 ng/ml) also induces a time-dependent increase in the intracellular lyso-PtdIns level of BACE cells. A similar increase in lyso-PtdIns occurs with CPAE cells (data not shown). These observations suggest that, in addition to its effect on phospholipase C, angiogenin activates an endothelial cell phospholipase A_2, as has been proposed for bradykinin (17, 18).

Effect of Angiogenin on Inositol Phosphate Release. Inositol phosphates were quantitated by column chromatography (16) after exposure of BACE cells to angiogenin (0.1 ng/ml). Without preincubation with lithium chloride, there is little, if any, characterizable increase in the amount of inositol phosphates. In contrast, after incubation for 30 min with 50 mM lithium chloride, an inhibitor of both inositol monophosphate phosphatase and inositol polyphosphate 1-phosphatase (19, 20), intracellular InsP_3 increases 20% (Fig. 4A), peaks at 15 s, and returns to baseline by 2.5 min.

The transient increase in InsP_3 is followed by an increase in inositol bisphosphate (InsP_2) (Fig. 4A, peak at 30 s) and subsequently inositol monophosphate (Ins$P$) (Fig. 4B, peak at 1 min). Ins$P$ remains elevated, presumably reflecting inhibition of inositol monophosphate phosphatase (19) by Li^+ and prolonged hydrolysis of PtdIns. It is clear that the inositol-specific phospholipase C is activated because (i) the successive peaks of InsP_3, InsP_2, and Ins$P$ follow precedent, and (ii) there is a large increase in counts in the Ins$P$ fraction, >4000 ± 350 cpm at 1 min. The latter is regarded as the most reliable indicator of inositol-specific phospholipase C activation (21). The endothelial cell agonist bradykinin promotes a 500% increase in InsP_3 (18); by comparison, the 20% increase induced by angiogenin appears small.

DISCUSSION

Activation of the Endothelial Cell Inositol-Specific Phospholipase C by Angiogenin. Angiogenin has been found to interact

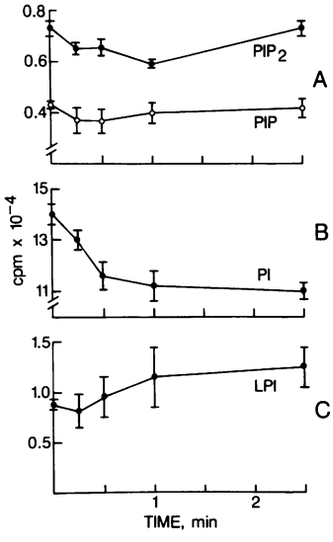

FIG. 3. Time course of angiogenin-induced inositol phospholipid metabolism in cultured BACE cells. Cells were prelabeled with *myo*-[^{3}H]inositol and exposed to angiogenin (0.1 ng/ml) at 37°C. Effect on intracellular (A) PtdInsP_2 (●) and PtdIns$P$ (○), (B) PtdIns, and (C) lyso-PtdIns.

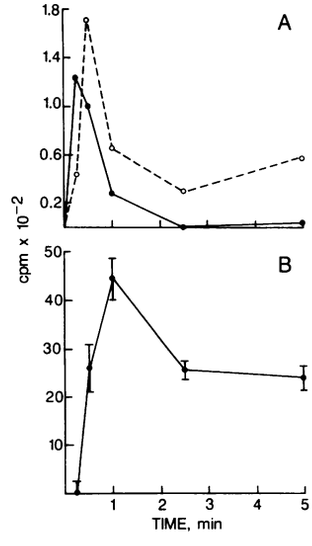

FIG. 4. Time course of angiogenin-induced inositol phosphate release in cultured BACE cells. Cells were prelabeled with *myo*-[^{3}H]inositol, incubated with lithium chloride (50 mM) for 30 min, and then exposed to angiogenin (0.1 ng/ml) at 37°C. (A) InsP_3 (●) (control, 700 ± 25 cpm) and InsP_2 (○) (control, 1055 ± 70). (B) Ins$P$ (control, 12,200 ± 1650).

with cultured endothelial cells to stimulate changes in the intracellular concentrations of second messengers. It induces a rapid 2-fold increase in the intracellular level of diacylglycerol, a putative cellular activator of protein kinase C (22). This is accompanied by a concomitant decrease in intracellular inositol-containing phospholipids and a transient 20% increase in InsP_3. Hydrolysis of PtdInsP_2 is an early event that leads to a small burst of InsP_3. PtdInsP_2 and InsP_3 return to the resting value within 2.5 min. In contrast, a decrease in PtdIns and increases in both diacylglycerol and Ins$P$ last for at least 10 min. The decrease in PtdIns could be due to (*i*) hydrolysis by phospholipase C, (*ii*) hydrolysis by phospholipase A_2 to lyso-PtdIns, or (*iii*) phosphorylation to PtdIns$P$ and PtdInsP_2. The threshold for diacylglycerol induction (0.001–0.01 ng/ml) is similar in all three endothelial cell lines examined. In contrast, the maximal response is variable, peaking at 1 ng/ml in arterial and at 0.1 ng/ml in capillary endothelial cells, but requiring higher concentrations of angiogenin in HUVE cells (100–1000 ng/ml). Reasons for this variability, apart from different species of origin of the cells, are not clear. With CPAE and BACE cells, the ability to induce an increase in diacylglycerol falls sharply with higher concentrations of angiogenin. Indeed, at concentrations of angiogenin comparable to those in normal human plasma, angiogenin induces only a small increase in intracellular diacylglycerol in arterial endothelial cells.

Although InsP_3 is postulated to release calcium from stores within the endoplasmic reticulum (23), we have been unable to detect an angiogenin-induced calcium flux when monitoring fura-2 fluorescence in cultured BACE monolayers (R.B., unpublished observations). Interferon stimulates InsP_3 formation in diploid fibroblasts (24) but without induction of a detectable calcium flux. The angiogenin-induced increase in InsP_3 is small (≈20%) compared to that reported for calcium mobilizing agonists such as bradykinin (≈500%) (18), and possibly insufficient to stimulate a detectable calcium flux in this cell line. This contrasts with the large increases in diacylglycerol and Ins$P$. After exposure of the endothelial cell to angiogenin, diacylglycerol appears to be the major intracellular messenger (Fig. 5).

The associated increase in lyso-PtdIns suggests that angiogenin activates an endothelial cell phospholipase A_2. However, a recent report failed to detect phospholipase A_2 activity in CPAE cell extracts (25) and cautions against taking lyso-PtdIns formation alone as evidence for activation of phospholipase A_2. Nevertheless, the activity of agonist-stimulated cell extracts was not examined, and it is possible that detectable phospholipase A_2 activity is seen only after exposure of intact cells to agonist.

Angiogenin-Stimulated Diacylglycerol Formation and Activation of the Diacylglycerol-Dependent Protein Kinase. Diacylglycerol activates protein kinase C, which phosphorylates critical proteins, potentiating the cellular response to the original agonist. Well known targets of protein kinase C are an 80-kDa protein of unknown function (26), the epidermal growth factor receptor (27, 28), and the Na^+/H^+ pump—either directly or indirectly (29–32). Although these responses are well characterized for mitogen activation of protein kinase C (33), stimulation of diacylglycerol formation by nonmitogens does not seem to evoke similar cellular reactions. Thus, interferon inhibits cell growth and, despite a well characterized increase in diacylglycerol (24, 34), none of the aforementioned "classic" responses to protein kinase C activation has been observed (35). Nevertheless, interferon-dependent protein kinase activities have been noted with mouse 3T3-F442A and 3T3-C2 fibroblasts. Interferon stimulation promotes cAMP-independent phosphorylation of a protein that is similar to the double-stranded RNA-dependent eukaryotic initiation factor 2α protein kinase (36), which regulates protein synthesis in rabbit reticulocytes (37, 38). It appears that with nonmitogens (such as angiogenin), protein kinase C activation phosphorylates and activates proteins that are crucial but as yet largely unidentified.

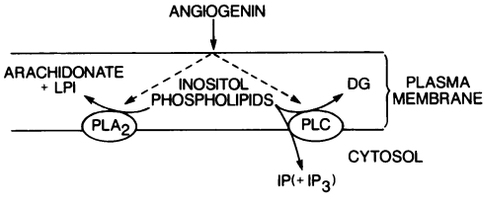

FIG. 5. Proposed endothelial cell response to angiogenin. The mechanism of transduction is not yet characterized, as indicated by dashed arrows. LPI, lyso-PtdIns; DG, diacylglycerol; PLA_2, phospholipase A_2; PLC, phospholipase C; IP, Ins$P$; IP_3, InsP_3.

Comparison of the Effect of Angiogenin and Phorbol Esters on Endothelial Cells. Although angiogenin stimulates endothelial cells to increase the intracellular level of diacylglycerol, the putative *in vivo* activator of protein kinase C, it does not induce changes in cell morphology. This contrasts sharply with the effect of the "artificial" activator of protein kinase C, phorbol 12-myristate 13-acetate, which promotes striking morphological changes in capillary endothelial cells (39). Recent work has shown, however, that phorbol esters elicit other cellular responses not known to be mediated by protein kinase C activation (40).

Protein kinase C activators—e.g., β-phorbol 12,13-dibutyrate—suppress the stimulation of capillary endothelial cell growth induced by an angiogenic endothelial mitogen (41). It was suggested that this response may operate when proliferating endothelial cells differentiate and organize into nongrowing tubes. The induction of diacylglycerol by angiogenin could similarly potentiate an endothelial cell response to angiogenic mitogens, such as fibroblast growth factor. However, mixtures of angiogenin and acidic fibroblast growth factor are neither additive nor synergistic in the chorioallantoic membrane assay (42).

Ribonucleolytic Activity of Angiogenin and the Cellular Response. The present data show that the ribonucleolytic activity of angiogenin is essential to elicit a cellular response. This is in agreement with earlier observations that placental ribonuclease inhibitor abolishes both the ribonucleolytic and angiogenic activities of angiogenin (43). In this respect, the endothelial cell response to angiogenin is similar to its response to thrombin, which promotes an increase in diacylglycerol by activating the inositol-specific phospholipase C in endothelial cells (44). In the latter case, the catalytic—proteolytic as contrasted with ribonucleolytic—activity is an absolute requirement for thrombin activation of endothelial cell phospholipase C (45).

Angiogenin interacts with both RNA—e.g., ribosomal RNA (5)—and proteins—e.g., tight binding to placental ribonuclease inhibitor (43). Both interactions require an intact active site (46). Attempts to identify specific angiogenin receptors on endothelial and many other cell lines have shown that angiogenin binds rapidly to the cell surface. However, extensive nonspecific binding (W. F. Heath, personal communication), presumably due to the exceptionally high pI (>9.5) of angiogenin (3) and the acidic nature of the cell surface, has made it difficult to characterize the low level of specific binding. It is hoped that further work will enable characterization of putative angiogenin receptors.

Relevance to *in Vivo* Angiogenesis. The relevance of the present data to the *in vivo* angiogenic activity of angiogenin is still in need of clarification. A simplistic explanation would propose that the principal reservoir of angiogenin is blood plasma, although other (including abnormal) reservoirs may yet be identified. Release of low concentrations of angiogenin from plasma, its normal physiological reservoir, could lead to

angiogenesis in response to tissue damage. Under such circumstances, it would activate cells and promote angiogenesis, presenting a classic example of organogenesis through an organizer gradient (47). Among other possibilities, angiogenin may occur in plasma complexed with an as yet unidentified inhibitor and be released by a subtle control mechanism. The function of circulating angiogenin, other than as a reservoir, is at present not clear; however, the high plasma concentration points to a potential role in homeostasis. If the angiogenin in plasma is active, the endothelial cells will be continually exposed to concentrations higher than that required to elicit a cellular response. This suggests that expression of a surface receptor, or some other process, must be rate limiting in the cellular response.

We thank Drs. Robert Shapiro and Stana Weremowicz for providing placental ribonuclease inhibitor and bromoacetate-modified angiogenin. This work was supported by funds from Hoechst, A.G., under agreements with Harvard University.

1. Fett, J. W., Strydom, D. J., Lobb, R. R., Alderman, E. M., Bethune, J. L., Riordan, J. F. & Vallee, B. L. (1985) *Biochemistry* **24,** 5480–5486.
2. Denèfle, P., Kovarik, S., Guitton, J.-D., Cartwright, T. & Mayaux, J. F. (1987) *Gene* **56,** 61–70.
3. Strydom, D. J., Fett, J. W., Lobb, R. R., Alderman, E. M., Bethune, J. L., Riordan, J. F. & Vallee, B. L. (1985) *Biochemistry* **24,** 5486–5494.
4. Shapiro, R., Riordan, J. F. & Vallee, B. L. (1986) *Biochemistry* **25,** 3527–3532.
5. St. Clair, D. K., Rybak, S. M., Riordan, J. F. & Vallee, B. L. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 8330–8334.
6. Leibovich, S. J., Polverini, P. J., Shepard, H. M., Wiseman, D. M., Shively, V. & Nuseir, N. (1987) *Nature (London)* **329,** 630–632.
7. Frater-Schroder, M., Risau, W., Hallmann, R., Gautschi, P. & Bohlen, P. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 5277–5281.
8. Folkman, J. & Klagsbrun, M. (1987) *Science* **235,** 442–447.
9. Shapiro, R., Strydom, D. J., Olson, K. A. & Vallee, B. L. (1987) *Biochemistry* **26,** 5141–5146.
10. Kurachi, K., Rybak, S. M., Fett, J. W., Shapiro, R., Strydom, D. J., Olson, K. A., Riordan, J. F., Davie, E. W. & Vallee, B. L. (1988) *Biochemistry*, in press.
11. Yin, E. T., Galanos, C., Kinsky, S., Bradshaw, R. A., Wessler, S., Luderitz, O. & Sarmiento, M. E. (1972) *Biochim. Biophys. Acta* **261,** 284–289.
12. Shapiro, R., Weremowicz, S., Riordan, J. F. & Vallee, B. L. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 8783–8787.
13. Voyta, J. C., Via, D. P., Butterfield, C. E. & Zetter, B. R. (1984) *J. Cell Biol.* **99,** 2034–2040.
14. Griendling, K. K., Rittenhouse, S. E., Brock, T. A., Ekstein, L. S., Gimbrone, M. A. & Alexander, R. W. (1986) *J. Biol. Chem.* **261,** 5901–5906.
15. Gonzalez-Sastre, F. & Folch-Pi, J. (1968) *J. Lipid Res.* **9,** 532–533.
16. Downes, C. P. & Michell, R. H. (1981) *Biochem. J.* **198,** 133–140.
17. Hong, S. L. & Deykin, D. (1982) *J. Biol. Chem.* **257,** 7151–7154.
18. Lambert, T. L., Kent, R. S. & Whorton, A. R. (1986) *J. Biol. Chem.* **261,** 15288–15293.
19. Hallcher, L. M. & Sherman, W. R. (1980) *J. Biol. Chem.* **255,** 10896–10901.
20. Inhorn, R. C. & Majerus, P. W. (1987) *J. Biol. Chem.* **262,** 15946–15952.
21. Irvine, R. F. (1986) in *Phosphoinositides and Receptor Mechanisms*, ed. Putney, J. W. (Liss, New York), pp. 89–108.
22. Nishizuka, Y. (1984) *Nature (London)* **308,** 693–698.
23. Berridge, M. J. & Irvine, R. F. (1984) *Nature (London)* **312,** 315–321.
24. Yap, W. H., Teo, T. S. & Tan, Y. H. (1986) *Science* **234,** 355–358.
25. Martin, T. W. & Wysolmerski, R. B. (1987) *J. Biol. Chem.* **262,** 13086–13092.
26. Rozengurt, E., Rodriguez-Pera, A. & Sinnet-Smith, J. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 7244–7248.
27. Davis, R. J. & Czech, M. P. (1984) *J. Biol. Chem.* **259,** 8545–8549.
28. Sinnett-Smith, J. & Rozengurt, E. (1985) *J. Cell Physiol.* **124,** 81–86.
29. Burns, C. P. & Rozengurt, E. (1983) *Biochem. Biophys. Res. Commun.* **116,** 931–938.
30. Vara, F., Schneider, J. A. & Rozengurt, E. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 2384–2388.
31. Owen, N. E. (1985) *J. Cell Biol.* **101,** 454–459.
32. Moolenaar, W. H., Tertoolan, L. G. & de Laat, S. W. (1984) *Nature (London)* **312,** 371–374.
33. Rozengurt, E. (1986) *Science* **234,** 161–166.
34. Yap, W. H., Teo, T. S., McCoy, E. & Tan, Y. H. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 7765–7769.
35. Mehmet, H., Morris, C. M. G., Taylor-Papadimitriou, J. & Rozengurt, E. (1987) *Biochem. Biophys. Res. Commun.* **145,** 1026–1032.
36. Petryshyn, R., Chen, J. J. & London, I. M. (1984) *J. Biol. Chem.* **259,** 14736–14742.
37. Farrell, P. J., Balkow, K., Hunt, T., Jackson, R. J. & Trachsel, H. (1977) *Cell* **11,** 187–200.
38. Levin, D. H. & London, I. M. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 1121–1125.
39. Montesano, R. & Orci, L. (1985) *Cell* **42,** 469–477.
40. Brooks, R. C., Morell, P., DeGeorge, J. J., McCarthy, K. D. & Lapetina, E. G. (1987) *Biochem. Biophys. Res. Commun.* **148,** 701–708.
41. Doctrow, S. R. & Folkman, J. (1987) *J. Cell Biol.* **104,** 679–687.
42. Fett, J. W., Bethune, J. L. & Vallee, B. L. (1987) *Biochem. Biophys. Res. Commun.* **146,** 1122–1131.
43. Shapiro, R. & Vallee, B. L. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 2238–2241.
44. Moscat, J., Moreno, F. & Garcia-Barreno, P. (1987) *Biochem. Biophys. Res. Commun.* **145,** 1302–1309.
45. Jaffe, E. A., Grulich, J., Weksler, B. B., Hampel, G. & Watanabe, K. (1987) *J. Biol. Chem.* **262,** 8557–8565.
46. Lee, F. S., Shapiro, R. & Vallee, B. L. (1988) *Biochemistry*, in press.
47. Spemann, H. & Mangold, H. (1924) *Wilhelm Roux Arch. Entwicklungsmech. Org.* **100,** 599–638.