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## Specific binding of angiogenin to calf pulmonary artery endothelial cells

(angiogenesis/pancreatic ribonuclease A/placental ribonuclease inhibitor)

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**ABSTRACT** Specific binding of angiogenin (ANG) to calf pulmonary artery endothelial cells was demonstrated. Cellular binding at 4°C of <sup>125</sup>I-labeled human recombinant ANG was time and concentration dependent, reversible, and saturable in the presence of increasing amounts of the unlabeled molecules. The interaction was shown to be specific since a large excess of unlabeled ANG reduced labeled ANG binding by >80%, whereas similar doses of RNase A, a structurally related protein, had no effect. Scatchard analyses of binding data revealed two apparent components. High-affinity sites with an apparent dissociation constant of  $5 \times 10^{-9}$  M were shown to represent cell-specific interactions. The second component, comprising low-affinity/high-capacity sites with an apparent dissociation constant of  $0.2 \times 10^{-6}$  M, was essentially associated with pericellular components. High-affinity ANG binding sites varied with cell density and were found on other endothelial cells from bovine aorta, cornea, and adrenal cortex capillary but not on Chinese hamster lung fibroblasts. Divalent copper, a modulator of angiogenesis, was found to induce a severalfold increase in specific cell-bound radioactivity. Placental ribonuclease inhibitor, a tight-binding inhibitor of both ribonucleolytic and angiogenic activities of ANG, abolished <sup>125</sup>I-labeled human recombinant ANG binding only in the absence of copper.

Angiogenin (ANG), a potent blood vessel-inducing polypeptide of 14 kDa, was first isolated from conditioned medium from human colon adenocarcinoma cells HT29 (1). However, it is not a tumor-specific product and has also been purified from human and bovine plasma (2, 3) and bovine milk (4). Moreover, ANG mRNA has been detected in human tumors and normal cells, such as epithelial cells, fibroblasts, and peripheral blood cells (5), as well as in various normal rat tissues and predominantly in rat adult liver (6). Levels of ANG and ANG RNA transcripts in tissues and fluids are not obviously related with an angiogenic state, suggesting that other mechanisms must control the potential angiogenic activity of ANG. ANG is a fairly well conserved molecule, since 65% of the amino acid sequence of human ANG (2, 7) is identical to that of bovine ANG isolated from milk, with most of the differences resulting from conservative substitutions (4).

Although ANG has 35% sequence identity (7) and an overall homology of 68% (8) with pancreatic RNase, it has been shown to be inactive in standard pancreatic RNase assays. However, ANG exhibits specific ribonucleolytic activity toward ribosomal and transfer RNAs (9-12). A possible physiological relevance of this enzymic activity is suggested by the fact that human placental RNase inhibitor

(PRI) (13-15) behaves as a potent antagonist of both the angiogenic and the ribonucleolytic activities of ANG (16, 17).

Unlike other angiogenic polypeptides, ANG alone has no known effect on cell proliferation but has been reported to modulate the mitogenic effect of certain conditioned media (18). However, its effect on capillary growth *in vivo* and its high concentration in plasma suggest that ANG may be involved in endothelium homeostasis. Moreover, recent reports have shown ANG-stimulated diacylglycerol formation and prostacyclin secretion in cultured endothelial cells (19, 20), suggesting the existence of specific cell-surface receptors. The studies presented here describe evidence of specific binding of <sup>125</sup>I-labeled ANG (<sup>125</sup>I-ANG) to endothelial cells.

### MATERIALS AND METHODS

**Materials.** Six different preparations of human recombinant ANG (rANG) were produced in *Escherichia coli* and purified as described (21). They migrated as a single band in SDS/PAGE (22). rANG differs from natural human angiogenin (nANG) in possessing N-terminal methionine instead of the natural N-terminal pyroglutamic residue (23), but it has been shown to be active as an angiogenic factor in the chorioallantoic membrane assay (21) and to induce blood vessel growth in the rabbit cornea (unpublished data). It exhibits characteristic ribonucleolytic activity (21). nANG was isolated from human plasma as described (2). Human PRI was obtained from Pharmacia.

**Cell Culture.** Calf pulmonary artery endothelial cells (CPAEs) (CCL209) and Dede Chinese hamster lung fibroblasts (CCL39) were obtained from the American Type Culture Collection and subcultured for <20 passages. CPAEs were grown in Eagle's minimum essential medium (GIBCO)/20% fetal calf serum (FCS) and CCL39 in Dulbecco's modified Eagle's medium (DMEM; GIBCO)/10% FCS. Bovine vascular aortic endothelial cells (ABAEs) were kindly provided by P. Böhlen (Institute for Cell Biology, Zürich) and cultured as described (24). Bovine capillary endothelial cells (BCEs), a gift from J. Folkman (Harvard Medical School, Cambridge, MA), were isolated from adrenal cortex and cultured on gelatin-coated tissue culture dishes (25). ABAEs and BCEs were maintained in DMEM/10% FCS containing glucose (1 g/liter). Bovine corneal endothelial cells (BECs) were grown from primary culture in DMEM/10% FCS as described (26). Culture media for ABAEs, BCEs, and BECs were supplemented with basic fibroblast growth factor (1 ng/ml) purified from bovine brain (27). Cells were maintained

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Abbreviations: ANG, angiogenin; rANG, human recombinant ANG; nANG, human natural ANG; ECM, extracellular matrix; ABAE, adult bovine aortic endothelial cell; BCE, bovine capillary endothelial cell; BEC, bovine corneal endothelial cell; CPAE, calf pulmonary artery endothelial cell; PRI, placental ribonuclease inhibitor; BSA, bovine serum albumin.

at 37°C in humidified 5% CO<sub>2</sub>/95% air. They were seeded at 5000 cells per cm<sup>2</sup> into multiwell plates (Falcon, Becton Dickinson) and incubated for 2 days before binding experiments.

**Extracellular Matrix (ECM) Production.** Cell-free ECM was obtained by lysis of the cell layer as described (28).

**Radiolabeling of Proteins.** ANG was labeled by the chloramine-T method (29). ANG was then desalted on a PD10 Sephadex G25-M column (Pharmacia) equilibrated with phosphate-buffered saline or 20 mM HEPES/0.14 M NaCl, pH 7.2, containing 1 mg of bovine serum albumin per ml (BSA; fraction V; Sigma). The specific activity varied from 80,000 to 120,000 cpm/ng (0.7–1 Ci/μmol; 1 Ci = 37 GBq) corresponding to ≈0.4 atom of iodine bound per molecule of ANG. Ribonucleolytic activity of iodinated ANG was checked on a large scale preparation of the molecule labeled with NaI containing 0.3% Na<sup>125</sup>I (Oris, Gif-sur-Yvette, France; 1.7 atoms of iodine bound per ANG molecule). RNase A (Sigma) was iodinated with a specific activity of ≈2 Ci/μmol.

**Binding Assay.** Assay conditions are described in the figure legends; unless otherwise noted, cell monolayers in multiwell plates were cooled to 4°C for 30 min before washing three times with binding buffer. Volumes were 0.25 ml per 2-cm<sup>2</sup> well. The standard binding buffer was HEPES buffer (20 mM HEPES/0.13 M NaCl/1 mg of BSA per ml, pH 7.2) containing 1 mM CaCl<sub>2</sub>/1 mM MgCl<sub>2</sub>/3 mM KCl/0.1 mM CuSO<sub>4</sub>. Cells were incubated at 4°C with <sup>125</sup>I-ANG in binding buffer for 4 hr on a shaking table, rinsed three times with the same buffer, and then washed once for 2 min at 4°C with washing buffer (binding buffer containing 0.6 M NaCl). Cells were solubilized overnight at 4°C with solubilization buffer [20 mM HEPES/10% (vol/vol) glycerol/1% Triton X-100/1 mg of BSA per ml, pH 7.2]. Residual extracellular components were then dissolved off the plastic wells with 4 M guanidine hydrochloride/2% Triton X-100/50 mM sodium acetate, pH 5.8. Radioactivity was measured with a LKB Minigamma counter at 80% efficiency. Each value is the mean of triplicate determinations. Nonspecific binding was defined as the amount of labeled ANG bound in the presence of the large excess of unlabeled ANG. Saturation experiments were carried out with increasing concentrations of labeled and unlabeled ANG and were analyzed according to the method of Scatchard (30) by the LIGAND fitting program (31).

## RESULTS

**Evidence for Cell-Specific Binding of <sup>125</sup>I-ANG to Endothelial Cells.** Previous attempts to establish the existence of cell-specific ANG receptors were made difficult by the marked tendency of this basic molecule, pI > 9.5 (1), to bind

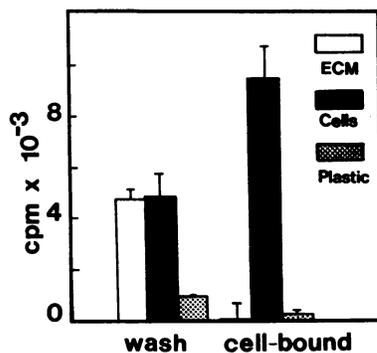


FIG. 1. Binding of <sup>125</sup>I-rANG to CPAEs that is competitive with unlabeled ANG (1000-fold excess). CPAEs, ECM, and plastic wells were incubated at 4°C with <sup>125</sup>I-rANG (0.35 nM; 540,000 cpm) in HEPES buffer/0.1 mM CuSO<sub>4</sub>, pH 7.2, rinsed four times, washed with 0.6 M NaCl washing buffer, and then solubilized.

Table 1. Binding of <sup>125</sup>I-rANG to various endothelial cells and CCL39 fibroblasts

Cells	Cell density, cells per cm <sup>2</sup>	Specific binding, fmol per 10 <sup>6</sup> cells
ABAE	12,500	123 ± 20
BCE	19,300	437 ± 28
BEC	18,800	65 ± 13
CPAE	16,800	174 ± 9
CCL39	75,000	ND

Cells were incubated at 4°C with 1.4 nM <sup>125</sup>I-rANG in binding buffer as in Fig. 1. All the cell types were studied at the same relative density (first half of the exponential growth phase). Only solubilized cells were considered. ND, not detectable.

to most surfaces (19). To minimize this extensive nonspecific binding, different factors have been studied such as carrier proteins and ionic conditions (data not shown). In addition, experimental factors that could promote ANG receptor expression or modulate ANG specific binding, such as cell density, cell type, and metal ion influence, were also tested. To differentiate between the different types of possible ANG interactions on cell monolayers, less than half-confluent CPAEs, their ECM, and empty plastic wells were incubated in parallel with <sup>125</sup>I-rANG at 4°C. It was shown that a wash with 0.6 M NaCl liberated ANG that bound with low affinity to ECM and to cells. Remaining cell-associated radioactivity was released by solubilization of the cells and was taken to represent high-affinity specific binding to cell-surface receptors (Fig. 1). Measurement of total recovered activity indicated that some ANG remained bound to the ECM. This radioactivity could be released by treating the residual ECM with guanidine hydrochloride buffer (data not shown). ANG cell-binding sites appeared to be related to cell density, since binding assays at 4°C on CPAEs cultured for 1, 2, or 3 days revealed a decrease in <sup>125</sup>I-rANG cell-specific binding as the cell density increased (792 ± 103, 428 ± 19, and 57 ± 6 fmol per 10<sup>6</sup> cells, respectively).

Under the same experimental conditions as with CPAEs, <sup>125</sup>I-rANG was also shown to bind specifically to other bovine endothelial cells from various sources such as aorta (ABAEs), adrenal capillary (BCEs), and cornea (BECs), but no significant cell-associated radioactivity was observed with CCL39 fibroblasts under the same conditions of confluency (Table 1).

**Influence of Metal Ions on Cell Binding of <sup>125</sup>I-rANG.** Preliminary assays yielded low cell-associated radioactivity.

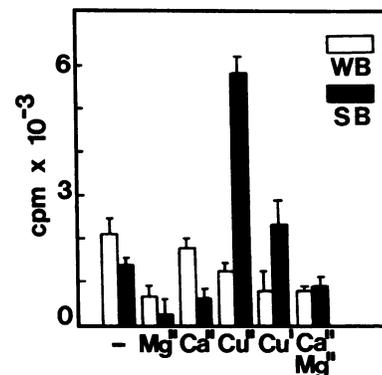


FIG. 2. Effect of metal ions on <sup>125</sup>I-rANG specific binding. CPAEs (422,000 cells per 10 cm<sup>2</sup>) were incubated at 4°C with <sup>125</sup>I-rANG (0.3 nM; 440,000 cpm) in HEPES buffer/BSA (2 mg/ml), pH 7.2, free of divalent cation (-) or containing 1 mM MgCl<sub>2</sub> (Mg<sup>II</sup>), 1 mM CaCl<sub>2</sub> (Ca<sup>II</sup>), 0.1 mM CuSO<sub>4</sub> (Cu<sup>II</sup>), 0.1 mM CuCl (Cu<sup>I</sup>), or 1 mM CaCl<sub>2</sub> and 1 mM MgCl<sub>2</sub> (Ca<sup>II</sup> Mg<sup>II</sup>). Radioactivity that is competitive with unlabeled ANG (560-fold excess) was released with washing buffer (WB) and in the solubilized portion (SB).

In view of the reported role of copper in the modulation of angiogenesis (32), the effect of copper ions on the binding of  $^{125}\text{I}$ -rANG to CPAEs was studied. As shown in Fig. 2, cell-specific binding increased in the presence of  $\text{Cu(II)}$  and  $\text{Cu(I)}$ . Other metal ions such as  $\text{Ca(II)}$  and  $\text{Mg(II)}$  induced a slight decrease in rANG-cell interactions. Maximum cell-specific binding was observed with  $100\ \mu\text{M}$   $\text{CuSO}_4$ , whereas  $^{125}\text{I}$ -rANG binding to pericellular components, released with the 0.6 M wash, decreased when copper was added (Fig. 3). For a limiting  $^{125}\text{I}$ -ANG concentration, as in Fig. 3,  $\text{Cu}^{2+}$  seemed to increase the tight binding at the expense of the weak one.

**Time Course of  $^{125}\text{I}$ -rANG-Cell Association and Dissociation.** Specific binding of  $^{125}\text{I}$ -rANG to CPAEs reached an equilibrium after 3 hr of incubation at  $4^\circ\text{C}$  (Fig. 4A). The reversibility of this interaction was demonstrated by an 80% displacement of total bound  $^{125}\text{I}$ -rANG in 10 min after the addition of a 500-fold excess of unlabeled rANG, whereas the observed spontaneous dissociation was much slower (Fig. 4B).

**Specificity of  $^{125}\text{I}$ -rANG-Cell Interactions.** Binding of  $^{125}\text{I}$ -rANG and  $^{125}\text{I}$ -nANG competed to the same extent with the unlabeled recombinant molecule (Fig. 5). Despite its sequence homology with ANG (23), RNase A did not interfere with ANG binding.

To detect potential artefactual binding due to the high pI ( $>9.5$ ) of ANG (1), binding experiments with  $^{125}\text{I}$ -RNase A were carried out under the same conditions but showed no cell-specific interaction (data not shown).

**Effect of PRI on  $^{125}\text{I}$ -rANG Binding.** When PRI, a tight-binding inhibitor of both ribonucleolytic and angiogenic activities of ANG (16), was preincubated in an equimolar ratio with  $^{125}\text{I}$ -rANG, the cell-associated radioactivity decreased by 77% in the absence of copper. This inhibition was blocked in the presence of  $100\ \mu\text{M}$   $\text{CuSO}_4$ . Considering the high cysteine content of PRI (14, 15), its stability toward metal ions was shown by incubating the protein in the presence of copper ions and subsequently chelating  $\text{Cu}^{2+}$  prior to the binding experiment.

**Characteristics of  $^{125}\text{I}$ -rANG Binding to CPAE Cultures.** Scatchard analysis (30) of  $^{125}\text{I}$ -rANG binding data from six independent experiments involving four different lots of rANG was resolved by the LIGAND program (31). This conglomerate analysis yielded two apparent families of interactions (Fig. 6). The apparent dissociation constant of the high-affinity binding sites was 5 nM and the average number of ANG molecules associated per cell was  $9 \times 10^5$ . This large number is not likely representative of ANG receptors and suggests that several ANG molecules could be bound to one specific binding site. The second component of the concave

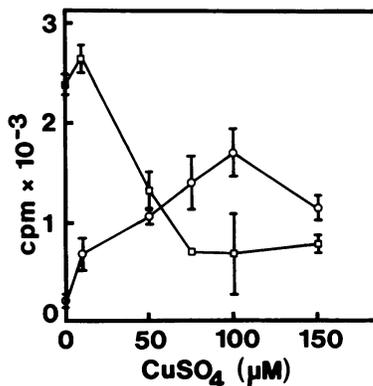


FIG. 3. Effect of  $\text{CuSO}_4$  on  $^{125}\text{I}$ -rANG specific binding. CPAEs ( $187,000$  cells per  $4\ \text{cm}^2$ ) were incubated at  $4^\circ\text{C}$  with  $^{125}\text{I}$ -rANG ( $0.25\ \text{nM}$ ;  $180,000\ \text{cpm}$ ) and increasing amounts of  $\text{CuSO}_4$  as described in Fig. 2.  $\square$ , Washing buffer;  $\circ$ , solubilization buffer.

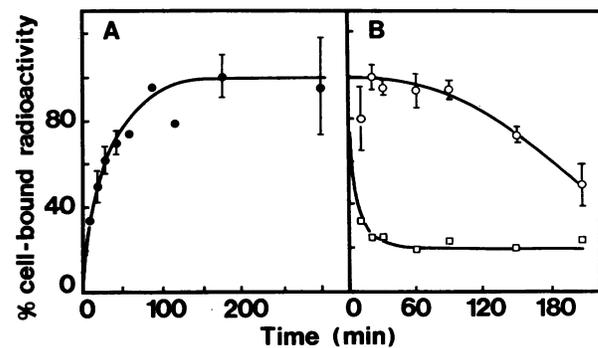


FIG. 4. Time course of  $^{125}\text{I}$ -rANG binding to CPAEs at  $4^\circ\text{C}$ . Only solubilized cells were considered. (A) Binding conditions: CPAEs,  $147,000$  cells per  $4\ \text{cm}^2$ ;  $^{125}\text{I}$ -rANG,  $0.3\ \text{nM}$  ( $220,000\ \text{cpm}$ ); 100% corresponds to  $3490\ \text{cpm}$  specifically bound ( $\text{SD} = 340\ \text{cpm}$ ;  $n = 3$ ). (B) Time course of dissociation at  $4^\circ\text{C}$  of cell-bound radioactivity. Binding conditions: CPAEs,  $61,000$  cells per  $4\ \text{cm}^2$ ;  $^{125}\text{I}$ -rANG,  $0.35\ \text{nM}$  ( $325,000\ \text{cpm}$ ). After removing the binding medium, dissociation was studied in the absence ( $\circ$ ) or in the presence ( $\square$ ) of  $0.17\ \mu\text{M}$  rANG. 100% corresponds to  $20,660\ \text{cpm}$  ( $\text{SD} = 3200\ \text{cpm}$ ;  $n = 6$ ). Error bars not indicated are smaller than symbol size.

Scatchard plot corresponding to low-affinity/high-capacity interactions involved several millions of molecules with an apparent dissociation constant of  $0.24\ \mu\text{M}$ . The large amount of bound ANG suggests associations with pericellular components.

Because of their large number, low-affinity interactions must be included in Scatchard analysis; however, saturation experiments focusing only on specific cell-bound radioactivity showed a concentration dependence of  $^{125}\text{I}$ -rANG-cell binding (Fig. 6 *Inset*) in the same range as the high-affinity sites deduced from the analyses of the total bound  $^{125}\text{I}$ -rANG (Fig. 6). Thus, the high-affinity component was considered to satisfy the criteria of an ANG receptor.

## DISCUSSION

ANG is one of the most potent inducers of neovascularization (1) when compared to other angiogenic polypeptides recently described, such as acidic and basic fibroblast growth factors, transforming growth factors  $\alpha$  and  $\beta$  (32), and tumor necrosis factor  $\alpha$  (33, 34). However, ANG has no known effect by itself on cell proliferation, migration, or other physiological events associated with angiogenesis. Its unusual ribonucle-

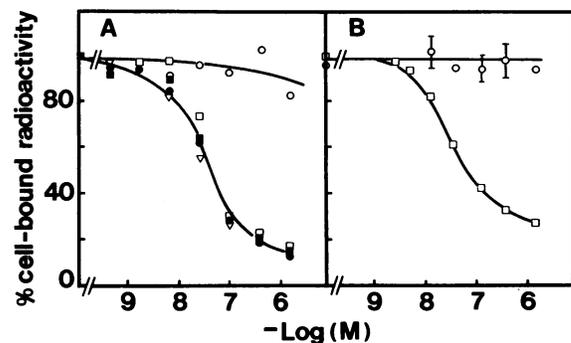


FIG. 5. Specificity of  $^{125}\text{I}$ -rANG-cell interactions. (A) CPAEs ( $56,000$  cells per  $2\ \text{cm}^2$ ) were incubated at  $4^\circ\text{C}$  with  $1.4\ \text{nM}$   $^{125}\text{I}$ -rANG ( $700,000\ \text{cpm}$ ) in the presence of increasing amounts of four different batches of unlabeled rANG ( $\square$ ,  $\nabla$ ,  $\bullet$ ,  $\blacksquare$ ) or RNase A ( $\circ$ ). 100% corresponds to  $47,950\ \text{cpm}$  ( $\text{SD} = 2400\ \text{cpm}$ ;  $n = 15$ ). (B) Parallel experiment with  $^{125}\text{I}$ -nANG ( $1.4\ \text{nM}$ ;  $600,000\ \text{cpm}$ ); CPAEs were  $94,000$  per  $2\ \text{cm}^2$ ;  $\square$ , unlabeled rANG;  $\circ$ , RNase. 100% corresponds to  $19,300\ \text{cpm}$  ( $\text{SD} = 1300\ \text{cpm}$ ;  $n = 15$ ). Only solubilized cells were considered.

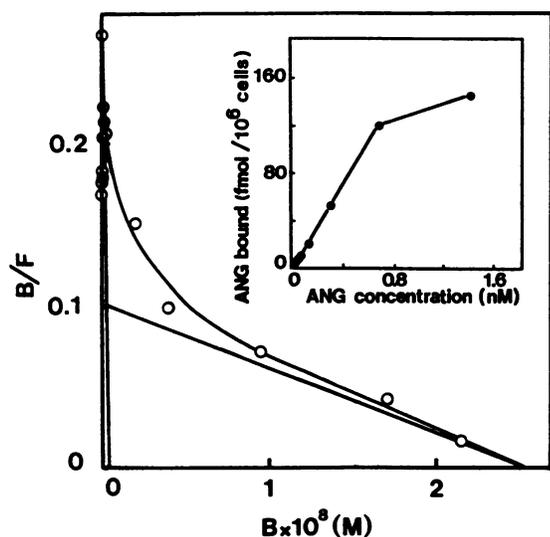


FIG. 6. Scatchard plot of the binding data of  $^{125}\text{I}$ -rANG to CPAEs. The curve was deduced by the LIGAND program (31) as the best fit according to its conglomerate analysis of  $^{125}\text{I}$ -rANG total binding data from six independent experiments involving four different preparations of rANG. Straight lines represent individual binding sites predicted from a two-site model (B, bound; F, free). (Inset) Concentration dependence of  $^{125}\text{I}$ -rANG specific cell binding to CPAEs. Only cell-associated radioactivity collected by Triton solubilization is presented.

olytic specificity toward ribosomal RNA (9, 10, 12, 35) and its angiogenic capacity appear to be interrelated since chemical modification or site-directed mutagenesis of amino acid residues involved directly (9, 36) or indirectly (37) in RNase catalysis have resulted in the abolition or enhancement of both the enzymic and the angiogenic activities. This apparent relationship suggests that RNA *in vivo* might be a target for ANG and points to a potential intracellular function of ANG. Recent studies have shown that ANG stimulates intracellular diacylglycerol formation and prostacyclin secretion in endothelial cells at concentrations that induce angiogenic responses in the chorioallantoic membrane assay (19, 20). These observations suggested that ANG might act via specific cell-membrane receptors.

In the present report, the existence of cell-specific receptors to ANG was indicated by direct binding studies of  $^{125}\text{I}$ -rANG or  $^{125}\text{I}$ -nANG to cultured endothelial cells.  $^{125}\text{I}$ -rANG was found to bind specifically to endothelial CPAEs as well as to ECM. Its cellular binding at 4°C was time and concentration dependent, was reversible, and was competed for by unlabeled rANG. In addition to overall amino acid sequence homology between ANG and pancreatic RNases (7, 23), the ribonucleolytic activity of ANG involves conserved essential lysine and histidine residues (11) whose chemical modification has been shown to abolish the capacity of ANG to induce an increase in CPAE cellular diacylglycerol (19). However, despite these structural similarities,  $^{125}\text{I}$ -RNase A did not bind specifically to CPAEs and the unlabeled molecule did not compete for ANG binding sites, thus emphasizing the specificity of the ANG-cell interactions described in this study.

Different types of ANG interactions with cell monolayers were differentiated by successive treatments with high ionic strength buffer, nonionic detergent to solubilize cell membranes, and a denaturing agent to release ECM components. Although Scatchard analyses did converge to indicate the presence of high-affinity binding sites with an apparent  $K_d$  in the nanomolar range, low-affinity/high-capacity interactions interfere to a large extent at equilibrium. Using the LIGAND program (31), conglomerate analysis of total bound  $^{125}\text{I}$ -

rANG showed two apparent types of interactions. The apparent dissociation constant of the high-affinity sites of  $5 \times 10^{-9}$  M is an order of magnitude higher than the concentration shown to induce diacylglycerol formation in CPAEs (19). A possible overestimation in the Scatchard analysis resulting from the large excess of low-affinity sites cannot be ruled out. However, this discrepancy may be due to the fact that only a small percentage of receptors need to be occupied to elicit a maximum second messenger response.

The regulation of receptors by cell density has been reported for growth factors such as nerve growth factor (38), epidermal growth factor (39), fibroblast growth factor (40), transforming growth factor  $\beta$ , and platelet-derived growth factor (41) and was considered to reflect their involvement in growth-related functions. Although the present data show that ANG cell-specific binding decreased with cell density, ANG had no effect on the growth of different types of endothelial cells (data not shown).

All the known properties of ANG (angiogenesis, ribonucleolytic activity, ability to activate endothelial cell phospholipase) have been shown to be fully inhibited by the RNase inhibitor isolated from human placenta (16, 19). In addition, PRI was shown in this work to antagonize  $^{125}\text{I}$ -rANG binding to CPAEs. These inhibitory effects clearly reflect the tremendously low  $K_i$  value of  $0.7 \times 10^{-15}$  M for the stoichiometric tight PRI-ANG interaction (17). RNase inhibitor in mammalian tissues (42) has been shown to be growth regulated (43), and its presence in plasma where it might neutralize circulating ANG has been demonstrated immunologically (44). These observations support the hypothesis that PRI may have a physiological role in the control of ANG function (8).

However, as described above, PRI was not able to antagonize  $^{125}\text{I}$ -rANG binding in the presence of copper, which was also shown to increase cell-specific binding. Although the mechanism is unclear, it has been proposed that copper can modulate angiogenesis (32). These findings suggest a tight regulation of ANG action in the process of angiogenesis and that PRI and copper might be involved in this regulation.

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